The role of *Campylobacter concisus* in enteric infections

A Thesis submitted in fulfillment of the requirements for the degree

of

Doctor of Philosophy

By

Eltaher Ahmed Elshagmani

BSc; M Lab Med

School of Applied Sciences

College of Science, Engineering and Health

RMIT University

August 2015

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Name: *Eltaher Ahmed Elshagmani*

Date: 03/08/2015

Acknowledgements

I would like to express my sincere gratitude to vital contributions given by the following people within the entire period of PhD candidature:

I am grateful for being given the opportunity by my senior supervisor, Dr. Taghrid Istivan, to being admitted into this PhD program. She certainly deeply effected the accomplishments leading to the results obtained from this research project. I would like also to recognise her for patiently support, kind assistance, persistent encouragement and great guidance throughout the entire period of my research. In particular, I am grateful to her for facilitating difficulties during the period of candidature.

I would like also to deeply acknowledge Prof. Margaret Deighton, Dr. Anna Walduck and Dr. Peter Ward, my associate supervisors for their assistance and support.

I would like to gratefully acknowledge the following Austin Hospital personnel who kindly participated and helped in the accomplishments of the inflammatory bowel disease (IBD) study. I would like to thank Dr. Peter Ward for his assistance and guidance in the study and in human ethics application. I would like to sincerely acknowledge Dr. Georgina Paizis for her involvement in establishing and planning the study, assistance in the preparation of the ethics application and with the enrolling and consent of IBD patients for the collection of clinical samples. I also greatly appreciate the help of Dr. Greg Lockrey and Dr. Christopher Leung for their involvement in the IBD patient study with the enrolling and consent of IBD patients for the collection of clinical samples. I would like to profoundly thank Ms Kirstin Hine (IBD nurse) for her assistance in sample collection from IBD patients by selecting the suitable patients from the clinician lists for the study, arranging the sample collection and preparing the paper work between the gastroenterology clinicians and myself. I would finally like also to thank all the nurses in Austin Hospital gastroenterology clinic who helped in the collection of samples during all endoscopy sessions.

I would like to thank RMIT statisticians (Dr. Adrian Schembri and Dr. Anthony Bedford) for their help and guidance particularly, in the determination of sample size for the IBD patient study.

I would like to thank the past and current students in Dr. Istivan's laboratory as they made the time in the laboratory very an enthusiastic and enjoyable place to work. I would like to thank Khaled Allemailem, Nahlah Almansour, Mohsina Huq, Jie Hu and Istiaque Ahmed for their invaluable friendship, assistance and encouragement.

I would like to thank the past and current students in the school of applied sciences (Bundoora) for their friendship and assistance, in particular Layla Alhasan, Mohamed Taha, Abdouslam Alsharif, Mohamed Said, Abdelwahab Badi, Abdulkarim Labaz, Abdulatif Mansur, Hala Almshawit, Thuy Nguyen, Rinu Thomas, Binu John.

I would like to gratefully admit the financial support that was granted to me by the Libyan government as a Foreign Scholarship to do a PhD in Australia.

Finally, I profoundly thank my family here in Australia and my extended family in Libya for their tremendous patience, support and encouragement, specially my parents, wife and children. I would like to thank my brothers and sisters as well.

Table of contents

List of Figures

List of Tables

List of Appendices

List of conference abstracts

1- Elshagmani, Eltaher. Allemailem, Khaled. Huq, Mohsina. Paizis, Georgina. G. Lockrey, Gregory. Leung, Christopher, Hine, Kirstin. Ward, Peter and Istivan, Taghrid. 'Incidences of *Campylobacter concisus* genetic subgroups in inflammatory bowel diseases (IBD)'. Campylobacter, Helicobacter and related organisms (CHRO) (18th) November, 2015, Rotorua, New Zealand.

2- Elshagmani, Eltaher. Allemailem, Khaled. Huq, Mohsina. Walduck, Anna. Ward, Peter and Istivan, Taghrid. 'The interaction between *Campylobacter concisus* and host epithelial cells *in vitro*'. Campylobacter, Helicobacter and related organisms (CHRO) (18th), November, 2015, Rotorua, New Zealand.

3- Elshagmani, Eltaher. Allemailem, Khaled. Huq, Mohsina. Paizis, Georgina. G. Lockrey, Gregory. Leung, Christopher, Hine, Kirstin. Ward, Peter. Walduck, Anna and Istivan, Taghrid. 'The prevalence of *Campylobacter concisus* in adults with inflammatory Bowel diseases'. ASM (42nd), July, 2014. Melbourne, Australia.

4- Eltaher Elshagmani, Khaled Allemailem, Mohsina Huq, Gena Gonis, Anna Walduck, Peter Ward, and Taghrid Istivan. 'The heterogenic nature of *Campylobacter concisus* in relevance to selected putative virulence genes'. BacPath (12th), September, 2013. Queensland, Australia.

5- Eltaher Elshagmani, Khaled Allemailem, Mohsina Huq, Gena Gonis, Anna Walduck, Peter Ward, Taghrid Istivan. 'Investigating the presence of putative virulence genes in *Campylobacter concisus* genome of oral and clinical isolates'. Campylobacter, Helicobacter and related organisms (CHRO) (17th), September, 2013, Aberdeen, Aberdeen.

6- Eltaher Elshagmani, Khaled Allemailem, Mohsina Huq, Gena Gonis, Anna Walduck, Peter Ward, Taghrid Istivan. 'A molecular study on *cjaC* gene region in *Campylobacter concisus* genome'. Campylobacter, Helicobacter and related organisms (CHRO) (17th). September, 2013, Aberdeen, Scotland.

7- Khaled Allemailem, Eltaher Elshagmani, Mohsina Huq, Gena Gonis, Anna Walduck, Taghrid Istivan. 'Investigating the genetic diversity of *Campylobacter concisus* clinical and oral strains'. ASM (41st), 2013, Adelaide, Australia.

8- Taghrid Istivan, Eltaher Elshagmani, Mohsina Huq, Gena Gonis, Andrew Daley, Peter Ward, Peter Coloe. '*Campylobacter concisus*: a heterogeneous emerging pathogen'. Campylobacter, Helicobacter and related organisms' (CHRO) (16th). September. Vancouver, Canada, 2011.

Abbreviations

Term	Meaning
λ	lambda phage DNA
μM	micromole
mM	millimole
μm	micrometer
μg	microgram
μl	microlitre
aa	amino acid
AFLP	amplified fragment length polymorphism analysis technique
AGRF	Australian Genome Research Facility
APS	ammonium persulfate
ATCC	American type culture collection
BHI	brain heart infusion broth
BLAST	basic local alignment sequence tool
bp	base pair
BSA	bovine serum albumin
CA	Columbia agar base
CB	Columbia broth
CD	Crohn's disease
CFU	colony forming units
CO2	carbon dioxide
°C	degrees Celsius
DMEM	dulbecco's modified Eagle's medium
ddH_2O	deionised distilled water.
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid

FBS	foetal bovine serum
g	gram
HBA	Columbia agar base with 5% horse blood agar
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HREC	Human Research Ethics Committee
h	hour
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
Kb	kilobase pairs
kDa	one thousand daltons
M	molar
mg	milligram
MLST	multilocus sequence typing
min	minute
MOI	the multiplicity of infection
ng	nanogram
N_2	Nitrogen
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	negative logarithm to the base ten of the concentration of hydrogen ions
qPCR	quantitative PCR
RCH	the Royal Children's Hospital
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N-Tetramethylethylenediamine
UC	ulcerative colitis

UV	ultraviolet
V	voltage
v/v	volume per volume
WGS	whole-genome shotgun contigs
w/v	weight per volume
WCLP	whole cell lysates protein

Summary

Campylobacter concisus is a fastidious hydrogen-requiring, Gram-negative, curved rod. This bacterium can be frequently isolated from the oral cavity of healthy individuals; however, the first isolation of *C. concisus* was in 1981 from a gum infection and it was then suggested to be a potential cause of gastroenteritis in children and immunocompromised adults. More recently, *C. concisus* has been associated with inflammatory bowel diseases (IBD) in children. However, some studies could not report a significant difference in the prevalence of *C. concisus* between IBD patients and healthy controls.

The potential pathogenic role of *C. concisus* in enteric infections is still not well investigated. Additionally, it has not been confirmed yet whether *C. concisus* is an opportunistic pathogen or if a certain group/genomospecies can cause human infections. Moreover, the virulence of *C. concisus* isolates from the oral cavity of healthy individuals and intestinal isolates has not been intensely investigated.

C. concisus has been grouped to two different genomospecies (A and B) on the basis of DNA-DNA hybridisation studies and PCR amplification of 23S rDNA. This study investigated the molecular diversity of potential pathogenic groups of *C. concisus* using two molecular techniques: PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (to amplify and analyse 16S rDNA), and a conventional PCR (to amplify several putative virulence genes including *cjaC, cjaA, dnaJ* and *zot*). These techniques were applied to *C. concisus* faecal and oral isolates. DGGE grouped *C. concisus* isolates into two new distinct groups. The putative virulence genes, *cjaC*, *cjaA* and *dnaJ*, were detected in the majority of *C. concisus* isolates, but there were DNA sequence variations, which could have led to PCR amplification failure in some *C. concisus* isolates. It was found that the location of *cjaC* in the genome of genomospecies A was different from its location in the genome of genomospecies B. Another

virulence gene (*zot*) encodes a tight junction toxin, could be only amplified from a few isolates.

An invasion assay was used to investigate the virulence of *C. concisus* using the human intestinal cell line INT407. Two *C. concisus* isolates from the oral cavity of healthy volunteers were identified as invasive isolates, while all tested faecal isolates from patients with diarrhoea were found to be non-invasive. Oral isolates obtained from healthy volunteers have not been previously reported to be more invasive than clinical intestinal isolates. Furthermore, *C. concisus* isolates possessing *zot* were unable to show significant invasion capability. In addition, semi-quantitative SYBR green assays were developed to evaluate the expression of the putative virulence genes (*cjaC, cjaA, dnaJ* and *zot*) in *C. concisus* grown on Columbia agar with the addition of horse blood (HBA), on Columbia agar (CA) only, maintained in tissue culture medium with or without INT407 cells. The expression of *cjaC* and *cjaA* was slightly enhanced when the bacterial cells were grown on CA compared with HBA. However, *dnaJ* expression was significantly reduced in bacterial cells sustained in the tissue culture medium only when compared with those maintained with INT407 cells growing in a similar tissue culture medium.

Another aspect of this study was to investigate the prevalence and possible association of particular *C. concisus* genomospecies in adults with IBD. A total of 350 intestinal biopsies (at least seven intestinal biopsies from each participant) were collected from 51 participants (IBD and control participants) attending the Gastroenterology Clinic at the Austin Hospital in Melbourne, Australia. Also from each participant, a gum and faecal samples were collected at the time of the biopsy collection. The presence of *C. concisus* was investigated by conventional culture and molecular techniques in all collected specimens. To detect the prevalence of *C. concisus* genomospecies B and *zot* directly from the clinical samples, new nested PCR techniques were developed. The direct detection of genomospecies A DNA in the

clinical samples was not included, as in the start of this study the whole genome of genomospecies A has not been published.

Overall, the prevalence of *C. concisus* DNA was significantly higher in biopsies of IBD patients (59.5%) than in biopsies of control participants (21.4%). Moreover, *C. concisus* was isolated for the first time from duodenal and ascending colonic biopsies. The prevalence of genomospecies B was significantly higher in IBD patients than in control participants using the nested PCR to detect this genomospecies in intestinal biopsies, faeces and gum samples. Among the *C. concisus* isolates cultured from gum samples, the prevalence of genomospecies B was also significantly higher in IBD patients than in control participants, while genomospecies A was more predominant in control participants. These findings suggested that genomospecies B might be associated with IBD. Furthermore, *zot* was not detected in most of the clinical isolates and there was no significant difference in its prevalence between IBD patients and control participants.

Collectively, the main results of this research project reinforced the extreme genetic diversity of *C. concisus* and more specifically between genomospecies A and B, on the basis of the putative virulence genes. In addition, it was shown that *C. concisus* invasive strains can exist in the oral cavity of healthy individuals. Gene expression assays for *cjaC, cjaA* and *dnaJ* were successfully developed in this study for the first time and it was shown that *dnaJ* was possibly involved in cellular invasion since its expression significantly reduced after exposure to tissue culture medium without INT407 cells. Furthermore, new PCR techniques were developed to detect genomospecies B and *zot* directly from clinical samples. The results suggested an association of *C. concisus*, in particular genomospecies B, with adults with IBD while no association of *zot* was recognised in the same group.

Chapter 1 : General introduction

1.1 *Campylobacter* species

Members of the *Campylobacter* genus are Gram-negative, curved or spiral rod shaped bacteria. Currently, they have been assigned to at least 25 species (Man 2011; Murray *et al.* 2009). Most of the members are motile with a polar flagellum at one end or two flagella located at each end (Man *et al.* 2010a; Vandamme *et al.* 2005). All *Campylobacter* spp. are extremely fastidious in utilising nutrients and require strictly anaerobic or microaerophilic conditions for growth. *Campylobacter* spp. normally colonise the gastrointestinal tract of mammals, reptiles, shellfish and birds (Debruyne *et al.* 2009; Inglis *et al.* 2006; Rossi *et al.* 2009; Tu *et al.* 2005).

1.1.1 *Campylobacter* species as pathogens

In recent decades, campylobacters have been considered as major causes of human gastroenteritis across the world (Altekruse *et al.* 1999; Tauxe 2001). The most common clinical features of enteric infections caused by campylobacters are watery or bloody diarrhoea, abdominal pain, fever, headache, anorexia and asthenia. These features can evolve after an incubation period of 2-3 days. In about 0.1% of cases, bacteraemia occurs (Skirrow *et al.* 1993), following translocation of the bacterial cells from the intestinal epithelial cells to the bloodstream. Immune deficient patients infected with *Campylobacter* spp. are more likely than patients with normal immunity, to develop systemic infections that require longer periods of hospitalisation (Sorvillo *et al.* 1991). The most common species to be related to systemic campylobacteriosis in humans is *C. fetus* (Blaser 1998), which could be related to its resistance to human serum factors (Kirk *et al.* 2015). Other species reported to be involved in

bacteraemia in humans include *C. insulaenigrae, C. upsaliensis* and *C. lari* (Chua *et al.* 2007; Lastovica 2006; Tauxe *et al.* 1985).

C. jejuni is a common cause of acute gastroenteritis in humans. Four million people including adults and children develop gastroenteritis due to *C. jejuni* each year worldwide (Allos 2001; Blaser *et al.* 1983; Leach 1997; Tauxe *et al.* 1987). Moreover, progression to serious diseases like reactive arthritis or Guillain-Barre syndrome can follow in a small number of patients who have acute infection (Altekruse *et al.* 1999). *C. coli* is the next most important *Campylobacter* spp. to cause gastroenteritis in humans, causing 4-18% of gastroenteritis cases caused by campylobacters (Friedman *et al.* 2004; Leach 1997; Valenza *et al.* 2010). In the last two decades, studies have shown the emergence of other *Campylobacter* spp. as potential causes of human enteric infection. These include *C. concisus, C. curvus, C. gracilis, C. lari* and *C. upsaliensis* (Maher *et al.* 2003; On 2001). Several recently discovered species (*C. helveticus, C. mucosalis* and *C. showae*) have not been recognised as pathogens, but more information is required on their possible pathogenic role (Man 2011). Recently, *C. concisus, C. hominis, C. showae* and *C. ureolyticus* were detected in intestinal biopsy specimens of children with Crohn's disease (CD) (Zhang *et al.* (2009), and *C. concisus, C. showae, C. gracilis, C. rectus* and *C. ureolyticus* were detected in faecal samples of children newly diagnosed with CD (Man *et al.* 2010b). Remarkably, *C. concisus* was the most predominant *Campylobacter* spp. in the intestine of children with CD (Man *et al.* 2010b; Zhang *et al.* 2009). Furthermore, *C. concisus* and *C. rectus* have been suggested to be associated with human periodontitis (Macuch *et al.* 2000; Tanner *et al.* 1981).

In animals, *Campylobacter* spp. including *C. jejuni, C. coli* and *C. hyointestinalis* are known to cause enteric infections in cattle and pigs, while *C. fetus* has been reported to be associated with abortion in sheep (Nachamkin *et al.* 2008).

Recent improvements in molecular techniques have led to an increase in the detection rates of these fastidious organisms from the human gastrointestinal tract and consequently more data has become available. Our understanding needs to be improved further on such fastidious bacteria based on developing the laboratory techniques to detect these species (in particular from the gastrointestinal tract), investigating their growth requirements and assessing their putative virulence factors.

1.2 *Campylobacter concisus*

C. concisus is a microaerophilic fastidious hydrogen-requiring, Gram-negative curved rod (Figure 1.1) that is normally found in the human oral cavity. It is motile with a single polar flagellum (Figure 1.2) (Paster *et al.* 1986). The cells are 0.5-1 µm in width and 2-6 µm in length (Figure 1.2) (Zhang *et al.* 2014). *C. concisus* is a small non-pigmenting asaccharolytic bacterium (Tanner *et al.* 1981) that grows slowly and requires enriched media. The G:C content of *C. concisus* DNA was found to be low (34-38%) (Tanner *et al.* 1981).

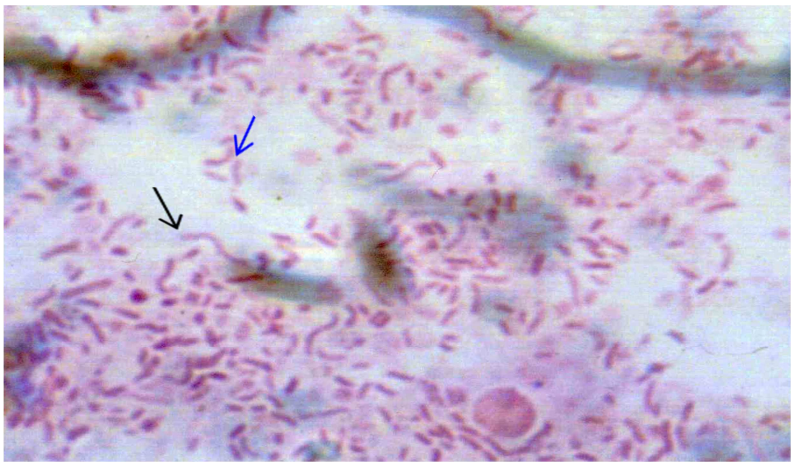


Figure 1.1: A Gram stained smear of *Campylobacter concisus* cells from 5 day old culture plate. The smear shows the short curved (blue arrow) and long curved (black arrow) cells. The magnification is 1000x (Istivan 2005).

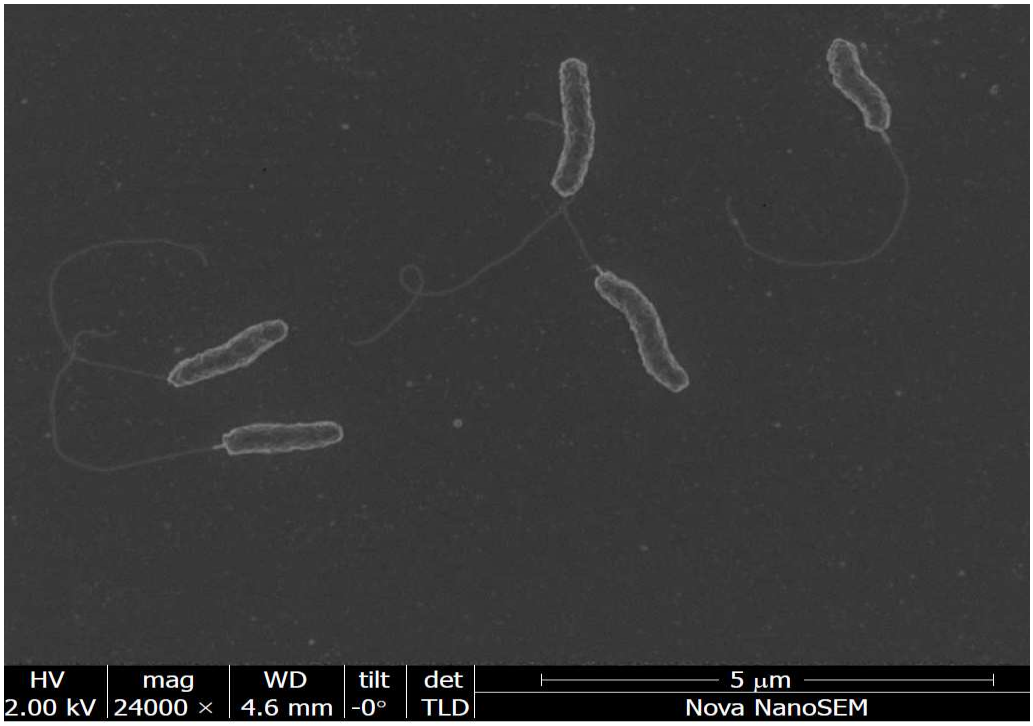


Figure 1.2: *Campylobacter concisus* cells under the electron microscope. The image shows the cells have a single polar flagellum (Ismail *et al.* 2012).

1.3 Association of *Campylobacter concisus* with human diseases

C. concisus has been isolated and detected by molecular means in clinical samples obtained from patients with different medical conditions; it has been detected in oral infections (Kamma *et al.* 2001; Socransky *et al.* 1998; Tanner *et al.* 1981), gastroenteritis (Istivan 2005; Istivan *et al.* 2004; On 2001), intestinal specimens of patients with IBD (Hansen *et al.* 2013; Mahendran *et al.* 2011; Man *et al.* 2010b; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009), and abscesses (de Vries *et al.* 2008; Johnson *et al.* 1987). It has been reported that the prevalence of *C. concisus* has been underestimated in clinical samples because suitable detection methods in clinical microbiology laboratories are limited, unlike the situation for *C. jejuni* and *C. coli* (Vandenberg *et al.* 2006). A better understanding of the relationship between *C. concisus* and human infections is required because its association with human diseases is still unclear.

1.3.1 Gastroenteritis

C. concisus is a common coloniser in the human oral cavity and has been isolated from several sites including teeth, gum and saliva. Thus, the oral cavity could be the main source of *C. concisus* to the gastrointestinal tract, as around 1-1.5 L of saliva is produced every day and passed to the gastrointestinal tract (Humphrey *et al.* 2001). However, several studies have found that the isolation rate of *C. concisus* from faeces was much lower than from saliva samples of healthy individuals (Man *et al.* 2010b; Zhang *et al.* 2010). These findings could be explained as the gastrointestinal tract may not be a suitable environment for *C. concisus* to colonise.

The initial isolation of *C. concisus* from the gastrointestinal tract was by Vandamme *et al.* (1989) using a standard culture technique and it was suggested to be associated with diarrhoea after being isolated from patients with persistent diarrhoea. Using the same

technique, *C. concisus* was subsequently isolated from faecal samples of 2.5-3% and <1.5% in children and adults with diarrhoea, respectively (Engberg *et al.* 2000; Istivan 2005; Lastovica 2009; Lastovica *et al.* 2000; Lauwers 1991; Lindblom *et al.* 1995). Using the filtration technique, the isolation rate increased to 6% and to 2.8% from faecal samples of patients with gastroenteritis and from healthy individuals respectively (Aabenhus *et al.* 2002; Engberg *et al.* 2000; Musmanno *et al.* 1998; Nielsen *et al.* 2013b; Van Etterijck *et al.* 1996). However, none of these studies have shown significant differences in *C. concisus* isolation rate between patients and healthy controls (Engberg *et al.* 2000; Nielsen *et al.* 2011a; Van Etterijck *et al.* 1996). Collado *et al.* (2013) used the filtration technique and failed to isolate *C. concisus* from either patients with gastroenteritis or healthy controls; however, this difference could be due to the use of smaller pore size filter 0.45-µm instead of the standard 0.65 µm filter (Collado *et al.* 2013).

Generally, molecular methods detected higher rates of *C. concisus* than culture methods due to possible its fastidious nature. Inglis *et al.* (2011) used two nested PCRs to amplify *C. concisus* DNA from faecal samples of patients with diarrhoea and healthy controls; the first PCR targeted the 23S rDNA gene, while the second PCR targeted the *cpn60* region. Curiously, the detection rate of *C. concisus* was significantly higher in healthy controls (57%) than in the patients (31%) (Inglis *et al.* 2011). The study concluded that the bacterium could be non-pathogenic. However, Collado *et al.* (2013) reported contradicting findings and the prevalence of *C. concisus* DNA in faeces of patients with gastroenteritis was higher than healthy controls.

The variations in the prevalence of *C. concisus* between the studies could be due to many factors such as a low bacterial load, age differences and sensitivity of different detection techniques (Newell 2005). Since, *C. concisus* has been detected in faeces of healthy controls, there remains uncertainty on the role of this bacterium as a pathogen in human gastroenteritis

(Engberg *et al.* 2000; Istivan 2005; Lastovica 2009; Lawson *et al.* 1998; Vandamme *et al.* 1989).

1.3.2 Infections of the oral cavity

The initial isolation of *C. concisus* from the oral cavity was from patients with gum disease. Three isolates were obtained from patients with gingivitis and another three were obtained from patients with periodontitis (Tanner *et al.* 1981; Tanner *et al.* 1987). The authors suggested that *C. concisus* might have a pathogenic role in oral cavity infections. Further evidence that *C. concisus* may be associated with periodontitis came from a study that detected higher numbers of *C. concisus* attached to teeth than other sites of the oral cavity of patients with periodontitis (Haffajee *et al.* 1984). *C. concisus* was also present in a higher numbers at bleeding sites of patients with progressive periodontitis lesions as compared to non-bleeding sites (Kamma *et al.* 2000a, 2000b; Kamma *et al.* 1994; Nakou *et al.* 1998). Others found higher levels of *C. concisus* antibodies in patients with periodontitis than in healthy controls (Ebersole *et al.* 1985; Taubman *et al.* 1992). Also supporting an association of *C. concisus* with periodontitis was the findings that *C. concisus* isolation rates were significantly higher when gingival crevicular fluid of patients was positive for aspartate aminotransferase (AST) compared to patients negative for AST in gingival crevicular fluid (Kamma *et al.* 2001). AST is normally confined to the host cell, but during cell damage or in cell death it is released into the extracellular space (Schmidt *et al.* 1985). The increase of the AST activity in gingival crevicular fluid is strongly associated with periodontal diseases (Chambers *et al.* 1991). *C. concisus* was also found to be more prevalent in the oral cavities of patients with gingivitis than in healthy individuals (Macuch *et al.* 2000; Moore *et al.* 1987).

In summary, several studies have reported the possible association of *C. concisus* with oral infections, but its association with these infections is still not well understood. To clarify links with the disease, studies should focus on the pathogenic role of specific *C. concisus* isolates from patients rather than the isolation rate from the oral cavity. It is unclear whether the presence of *C. concisus* in the site of the infection in the oral cavity is due to its involvement in the infections or secondary colonisation of an infected site.

1.3.3 Detection of *C. concisus* at other sites

The first isolation of *C. concisus* from a site other than the oral cavity or lower intestinal tract was from a diabetic foot ulcer (Johnson *et al.* 1987). Following that, Cox *et al.* (2003) detected *C. concisus* RNA in synovial fluid of patients with reactive and post infectious arthritis. Remarkably, the investigators could not detect the common *Campylobacter* spp. that have been reported to be related to reactive arthritis such as *C. jejuni* and *C. coli* (Cox *et al.* 2003).

In oesophageal biopsies collected from patients with Barrett's oesophagus (BO), the most prevalent *Campylobacter* spp. detected using PCR were *C. concisus* and *C. rectus*, while these species could not be detected in healthy controls. Hence, it was suggested that these campylobacters in BO patients may play a significant role in initiation, maintenance or exacerbation of the disease and progression to induce adenocarcinoma formation (Macfarlane *et al.* 2007). Furthermore, in oesophageal bacterial biofilms, Blackett *et al.* (2013) used the culture technique to show that *C. concisus* was the most predominant species and was present in a higher proportion in BO patients (42.2%) than in healthy controls (12.8%). However, it is not clear whether *C. concisus* was present in BO samples as one of the normal microbiota or it had a special role to play in the disease.

In gastric fluid samples collected by gastroscopy from patient with gastritis, *C. concisus* was found to be the highest transcriptionally active bacterium in the stomach. However, the study did not include healthy controls (von Rosenvinge *et al.* 2013). Thus, it is not clear whether the source of RNA in the gastric fluid was from the bacterial colonisation in the stomach or in the oral cavity.

Furthermore, *C. concisus* was isolated as part of the poly-microbial flora (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Prevotella* spp. and *Peptostreptococcus anaerobius*) in a brain abscess in a 65 year old man who had a post-craniotomy for a maxillary sinus carcinoma (de Vries *et al.* 2008). The source of infection it seemed to be from the oral cavity.

In summary, *C. concisus* has been isolated from several unusual locations of the human body other than the oral cavity or the lower intestinal tract. However, in all of these sites, *C. concisus* was detected in combination with other commensals which has made it difficult to confirm its role in the infections rather than being a contaminant due to its colonisation in the oral cavity. Consequently, *C. concisus*, if involved in these types of human infections, could be part of the poly-bacterial flora rather than causing an individual infection.

1.3.4 Immune deficiency diseases

A few studies investigated the prevalence of *C. concisus* in different sites of immunocompromised patients. A large study that included 11550 faecal samples from different patient groups found 70% of subjects that had *C. concisus* were immunocompromised (Aabenhus *et al.* 2002). Moreover there was a significantly higher prevalence of *C. concisus* in sub-gingival biofilms of HIV patients with detectable plasma viral load than in a control group of HIV patients with undetectable plasma viral load (Pereira *et al.* 2014). The higher association of *C. concisus* with these patients could be due to the

immune deficiency state, rather than microorganism characteristics as *C. concisus* isolates obtained from faeces and intestinal biopsies were susceptible to normal human serum *in vitro,* suggesting that normal human serum is protective (Kirk *et al.* 2015).

1.4 Inflammatory bowel diseases (IBD) in humans

IBD generally refers to two major chronic incurable diseases; Crohn's disease (CD) and ulcerative colitis (UC). Clinically, IBDs are usually chronic, idiopathic relapsing ailments that can occur at any site of the gastrointestinal tract (Podolsky 2002). The onsets of CD and UC can occur at all ages; however, CD is more likely to present during early adulthood while UC is more likely to present in late adulthood. CD is characterised by transmural inflammatory lesions that can spread via the mucosa into underlying serosa, while in UC the inflammation commonly occurs in the colon and rectum with ongoing inflammation in the sub mucosa (Colletti 2004; Podolsky 2002).

Histopathologically, CD lesions consist of tight granulomas formed by giant cells, macrophages, and epithelioid cells, while in UC the number of polymorphonuclear cells in the lamina propria and the intestinal crypts are elevated leading to abscess formation (Colletti 2004). The main distinguishable diagnosis between IBD and acute self-limited gastrointestinal tract mucosal infections is based on whether the infiltration of inflammatory cells in intestinal crypts are branching; whether the layer of intestinal epithelial cell change is acute or chronic; and whether granulomas are present. Also, the symptoms of IBD typically consist of acute episodes each lasting for several weeks (Colletti 2004; Hanauer 2006).

Over the past two decades, the number of IBD patients requiring hospitalisation has significantly increased, resulting in increased financial burden for that health services (Sonnenberg 2010). A study conducted in Greater Geelong, Victoria found the annual incidence rates of IBD in Australia was one of the highest in the world (Wilson *et al.* 2010).

It has been suggested that IBD depends on the involvement of several factors including the environment, genetic background, immune system and intestinal commensals (Fiocchi 1998; Podolsky 2002; Sartor 2008). In particular, several bacterial species were suggested to be associated with CD including *Helicobacter* spp., *Bacteroides* spp., *Mycobacterium avium* subspecies *paratuberculosis* and *Campylobacter* spp. (Bohr *et al.* 2004; Man *et al.* 2008; Zhang *et al.* 2006; Zhang *et al.* 2009). It was suggested that the overlying bacteria at the intestinal mucosa may cause initial damage to mucosal cells, facilitating penetration of other organisms through the mucosal layer, and triggering inflammatory markers of the host cell (O'Rourke *et al.* 2001).

1.5 Association of *C. concisus* with IBD

A few studies have investigated a possible association of *C. concisus* with IBD, based on the detection of *C. concisus* in intestinal biopsy, faecal, and saliva samples of patients (Tables 1.1 and 1.2).

1.5.1 Detection of *C. concisus* in the intestinal biopsies of patients with IBD

The detection rate of *C. concisus* DNA in intestinal biopsies varied widely between studies in IBD patients (33%-69%) and in healthy controls (2%-38%) (Tables 1.1 and 1.2). Initially, Zhang *et al.* (2009) found the prevalence of *C. concisus* DNA in colonic biopsies of children newly diagnosed with CD was significantly higher when compared with a healthy control group. The authors suggested that *C. concisus* may play a major role in the development of CD in young children (Zhang *et al.* 2009). A strain isolated from the intestinal biopsy of a CD patient was later named *C. concisus* UNSWCD (Kaakoush *et al.* 2012).

Table 1.1: The percentage of *C. concisus* detection in clinical specimens collected from patients with IBD and control participants, (adopted from Kaakoush *et al.* (2012))**.**

Disease	Recruits	Specimens source	PCR type	Primer set	Patients (%)	Controls (%)	Study
IBD	Children	Intestinal biopsy	One-step	Not stated	58.3	42.3	Hansen *et al.* (2011)
IBD	Adults	Intestinal biopsy	Nested	C418/C1228 Concisus	68.0*	36.0	Mahendran *et al.* (2011)
IBD	Children	Intestinal biopsy	One-step	C418/C1228	38.6	38.1	Hansen *et al.* (2013)
IBD	Adults	Faeces	One-step	16S rDNA	21	9.0	Tankovic *et al.* (2009)

IBD: inflammatory bowel disease.

CD: Crohn's disease.

UC: ulcerative colitis.

*: there was a significant difference between the patients and controls.

C418/C1228 & Concisus primer sets: targeting 16S rDNA.

Table 1.2: The prevalence of *C. concisus* in CD and UC patients, and control participants, (adopted from Kaakoush *et al.* (2012)).

Disease	Recruits	Specimens source	PCR type	Primer set	Patients (%)	Controls (%)	Study
CD	Children	Intestinal biopsy	One-step	C418/C1228	53.0*	2.0	Zhang *et al.* (2009)
CD	Children	Intestinal biopsy	One-step	Not stated	66.7	42.3	Hansen *et al.* (2011)
CD	Adults	Intestinal biopsy	Nested	C418/C1228 Concisus	67.0	36.0	Mahendran *et al.* (2011)
CD	Children	Intestinal biopsy	One-step	C418/C1228	44.8	38.1	Hansen *et al.* (2013)
UC	Children	Intestinal biopsy	One-step	Not stated	37.5	42.3	Hansen *et al.* (2011)
UC	Adults	Intestinal biopsy	Nested	C418/C1228 Concisus	33.3*	10.8	Mukhopadhya *et al.* (2011)
UC	Adults	Intestinal biopsy	Nested	C418/C1228 Concisus	69.0*	36.0	Mahendran *et al.* (2011)
UC	Children	Intestinal biopsy	One-step	C418/C1228	30.8	38.1	Hansen *et al.* (2013)
CD	Children	Faeces	Nested	C418/C1228 Concisus	65.0*	35.0	Man *et al.* (2010b)
CD	Children & Adults	Saliva	Nested	C418/C1228 Concisus	100.0	97.0	Zhang *et al.* (2010)

IBD: inflammatory bowel disease.

CD: Crohn's disease

UC: ulcerative colitis

*: there was a significant difference between the patients and controls.

C418/C1228 & Concisus primer sets: targeting 16S rDNA.

In a later study on adults with CD and UC, *C. concisus* DNA was detected by nested PCR in biopsy samples collected from four intestinal anatomic sites (ileum, caecum, descending colon and rectum), while bacterial isolation was only preformed on a caecal biopsy (Mahendran *et al.* 2011). The prevalence of *C. concisus* was found significantly higher in biopsies of patients with UC than in controls, but unlike the study in children with CD (Zhang *et al* (2009), there was no significant difference in the prevalence of *C. concisus* between CD patients and healthy controls. The authors stressed that the possible involvement of *C. concisus* in UC and CD requires confirmation with more investigations to be undertaken. Mukhopadhya *et al.* (2011) reported a significantly higher *C. concisus* DNA prevalence in colonic biopsies of adults with UC than in controls, using similar nested primer sets, although the precise colonic anatomic site was not stated. In contrast, other studies failed to show significant differences between patients with IBD and healthy controls in the detection rate of *C. concisus* DNA in colonic biopsies (Hansen *et al.* 2013; Hansen *et al.* 2011). The conclusion of these studies was that *C. concisus* was not strongly associated with IBD.

1.5.2 Detection of *C. concisus* in faecal samples of patients with IBD

Man *et al.* (2010b) found the prevalence of *C. concisus* DNA in faecal specimens of children newly diagnosed CD was significantly higher than in non-IBD patients and healthy controls using nested PCR (Table 1.2). This study suggested that there was a role of *C. concisus* in paediatric CD; nonetheless, the mechanisms of how *C. concisus* contributed and initiated CD were unclear. The study strengthened the suggestion previously made by Zhang *et al* (2009) on the role of *C. concisus* in IBD; however, Tankovic *et al.* (2009) could not show the statistical significant difference in the prevalence of *C. concisus* DNA in faecal samples between IBD patients and a control group (Table 1.1).

1.5.3 Detection of *C. concisus* in the oral cavity of patients with IBD

A single study performed by Zhang *et al.* (2010) investigated the prevalence of *C. concisus* in human saliva collected from IBD patients and healthy controls. *C. concisus* was isolated from 85% in patients with CD, 100% in UC and 75% in healthy controls. The detection rate of *C. concisus* by PCR was 100% in IBD (CD and UC) and 97% of healthy controls (Table 1.2). There were no significant differences in prevalence of *C. concisus* between the patients and healthy controls, or between males and females in the prevalence of *C. concisus* (Zhang *et al.* 2010).

1.5.4 Human immune responses against *C. concisus* in IBD

Little is known about specific immune response against *C. concisus.* Recently, 37 immunoreactive proteins of *C. concisus* strain UNSWCD were identified by Western blotting analysis in sera collected from patients with CD. Only three of these (flagellin B, ATP synthase F1 alpha subunit, and outer membrane protein 18) were recognised by the sera of all patients studied. Six of the immunoreactive proteins were associated with host cell adhesion (Kovach *et al.* 2011). Moreover, it was found that the level of *C. concisus* specific immunoglobulin G (IgG) in patients with CD was significantly higher than in healthy controls (Zhang *et al.* 2009).Thus, it was suggested that *C. concisus* is able to pass the intestinal mucosa to initiate an immune response (Kovach *et al.* 2011). However, *C. concisus* could also pass through the epithelial cells of the oral cavity and its antigens might be exposed to the immune system. Hence, investigations of whether the human immune response was generated from intestinal or from oral *C. concisus* colonisation are required.

1.5.5 Summary of IBD studies

Several studies using PCR reported a significantly higher rate of detection of *C. concisus* in the intestinal biopsies of IBD patients than in healthy controls; however, other studies failed to detect such a difference. This difference could be due different techniques and some studies used one-step PCR, while the other studies used a nested PCR approach. Nested PCR is more sensitive than the one-step PCR and indeed most of the studies using nested PCR showed a significant difference in detection of *C. concisus* in IBD patients compared with controls (Tables 1.1 and 1.2). The use of different primer sets could also affect the detection rate, due to the variations in DNA sequences within *C. concisus* strains. Finally, if more intestinal anatomic sample sites are used, this may increase the detection rate.

In contrast, the rate of isolation of *C. concisus* in patients with IBD has been <11% and *C. concisus* has not been isolated from healthy controls to date. Thus, all statistical analysis methods were based on PCR detection results. The isolation rate has remained constant over time, which could be due to colonisation in the intestinal tract in very small numbers, or the bacterium may exist in a viable but non-culturable form. It is also possible that *C. concisus* in the intestinal tract of IBD patients is derived from the mouth flora, and the number of bacterial cells colonising the intestinal tract increases due to pre-existing inflammation.

All the studies that detected *C. concisus* in intestinal biopsies were collected at one anatomic site of the colon in children, with the exception of a single study that recruited adults (Mahendran *et al.* 2011) that included four anatomic sites of the colon. Thus information is lacking on the presence of *C. concisus* in all intestinal anatomic sites. Moreover, there is no information with respect to *C. concisus* groups associated with IBD. Furthermore, it is unclear whether the intestinal strains are similar to the strains that colonise the mouth of the same person at the same time. Serological studies could improve our understanding of the bacterial involvement in IBD, in particular quantifying immunoglobulin M (IgM) by enzyme-

linked immunosorbent assay (ELISA) instead of IgG (as in past studies) in serum of patients with *C. concisus* isolated from biopsies, as this approach might strengthen the association of *C. concisus* with IBD, and determine whether the *C. concisus* antibodies were produced as result of the bacterial load in the oral cavity or from gut infections.

1.6 Colonisation of the oral cavity of healthy individuals with *C. concisus*

C. concisus is found significantly higher in numbers on the surface of permanent than deciduous teeth in healthy children with mixed dentition (Kamma *et al.* 2000a). Furthermore, it was isolated more frequently from molars than incisors in healthy children with primary dentition (Kamma *et al.* 2000a). Isolation of *C. concisus* from faeces of healthy individuals was very low compared with the oral cavity (Cornelius *et al.* 2012; Engberg *et al.* 2000; Nielsen *et al.* 2013a), suggesting that the intestinal tract is not a primary site of carriage. Interestingly, smokers were found to have a higher colonisation number of *C. concisus* than non-smokers in their oral cavity (Kamma *et al.* 1997; Kamma *et al.* 1999), which is possibly due to the potential risks of smoking associated with progression of gum diseases.

1.7 *C. concisus* in animals and in animal source foods

Several campylobacters have been detected in samples from mammals and birds but *C. concisus* has not been reported as a normal coloniser in any healthy animals (Chaban *et al.* 2010; Vandamme *et al.* 2005). *C. concisus* was detected in saliva of a 2 year old cat with gum infection, while the bacterium could not be detected in healthy dogs (Petersen *et al.* 2007). Moreover, *C. concisus* was detected in faeces of diarrheic dogs, while again none of the healthy dogs were positive for *C. concisus* (Chaban *et al.* 2009; Chaban *et al.* 2010). To date only a single study has detected *C. concisus* in faecal samples collected from broiler chickens with a proportion of 6.4%. This study suggested that chickens could be a reservoir for *C.*

concisus (Kaakoush *et al.* 2014). However, the study did not attempt to isolate *C. concisus* by culture to determine whether the chickens had viable bacterial cells in the gut.

Furthermore, using culture technique, *C. concisus* was isolated from retail fresh meat of chickens, beef and pork in low percentages (<10%) (Lynch *et al.* 2011; Scanlon *et al.* 2013) and also from raw cow's milk (Serraino *et al.* 2013). These examples of detection of *C. concisus* in animals, retail fresh meat or milk may have resulted from cross-contamination from humans as the very limited studies available have failed to detect *C. concisus* colonisation in healthy animals.

Although *C. concisus* was detected in humans and animals, no studies have sought its detection in plants that are a source of food for humans such as fruit and vegetables. These studies are required because plants might be a reservoir of *C. concisus* with the potential to transmit to the human gastrointestinal route. Therefore, it is likely that from the collective information of these studies, the human oral cavity is the primary reservoir of *C. concisus* as it commonly found there in healthy individuals.

1.8 Laboratory diagnosis, isolation and detection of *C. concisus* in clinical specimens

Since *C. concisus* is usually present in mixed culture with other organisms, including commensals, techniques using filtration and molecular techniques are more reliable than standard culture.

1.8.1 Cultivation and incubation conditions of *C. concisus*

C. concisus is routinely cultivated on Columbia agar base or blood agar base supplemented with 5-6% defibrinated horse blood (HBA) in a mixture of gases consisting of 7% H_2, 7% CO_2, 7% O_2 and ~79% N_2 in an anaerobic jar at 37 °C (Istivan *et al.* 2010; Istivan *et al.* 2004;

Lee *et al.* 2014). Microaerophilic growth conditions can also be generated by evacuating an anaerobic jar to -7 bar and then adding gas with a mixture of 10% H_2, 10% CO_2 and ~80% N_2 (Istivan *et al.* 2010; Lee *et al.* 2014). *C. concisus* appears on HBA as colonies measuring 1-2 mm in diameter, round, entire, semi translucent and grey in colour (Istivan 2005).

1.8.2 The isolation of *C. concisus* from clinical samples

There is no a standard technique for *C. concisus* isolation from faeces, saliva or tissue. However, the most common technique used for *C. concisus* isolation from faeces is the 'Cape Town protocol', which involves filtration of samples onto enriched media such as HBA without antibiotics (Engberg *et al.* 2000; Lastovica *et al.* 2000). The faecal sample is initially suspended in liquid medium or phosphate buffered saline (PBS) at 1:2 to 1:10; then, 4-5 drops are placed on a cellulose acetate filter (pores size 0.65 μm) positioned on HBA. The suspension is soaked in the filter for approximately 10 min. The filter was then discarded and the plate was incubated for 3-5 days (Istivan *et al.* 2010).

Tissue samples such as intestinal biopsies are spread on HBA containing 10 mg/ml of each trimethoprim and vancomycin prior to incubation under suitable growth conditions for *C. concisus* (Mahendran *et al.* 2011; Zhang *et al.* 2009). Alternatively, a two-step enrichment-filtration method can be used (Kaakoush *et al.* 2011b) as follows: in step 1, the biopsy is incubated in enrichment broth containing 3 ml of Ham's F-12 medium with foetal bovine serum (5% FBS) and 10 μg/ml of vancomycin; then, incubated under *C. concisus* growth conditions for 48 h. Step 2 consists of filtration of 200 μl from the growth mixture obtained from step 1 onto HBA containing 10 μg/ml of vancomycin; then, incubation under *C. concisus* growth conditions for 2-4 days (Kaakoush *et al.* 2011b).

Isolation of *C. concisus* from saliva was achieved by streaking 6 μl of saliva on HBA containing 10 μg/ml vancomycin and incubation under suitable growth condition for 3 days.

Then the mixed bacterial growth was filtered using cellulose acetate filter (pores size 0.65 µm) on a fresh HBA plate and incubated for 2 days (Zhang *et al.* 2010). However, this method might not reflect the original *C. concisus* load in saliva because *C. concisus* may grow during both incubation periods. Commensals that are resistant to vancomycin could also compete and reduce the growth of *C. concisus*.

Although most previous studies have used the cellulose acetate filter (pore size 0.65 µm), Nielsen *et al.* (2013b) recently reported that an alternative polycarbonate filter (pore size 0.60 µm) led to a significantly greater isolation rate for *C. concisus* from faecal samples (Nielsen *et al.* 2013b).

1.8.3 Detection and confirmation of *C. concisus* by molecular methods

For a bacterium of fastidious nature and slow in growth like *C. concisus*, molecular techniques can improve detection and identification in clinical samples. However, genetic variations should be considered in these detection methods otherwise some strains might be missed.

1.8.3.1 Direct detection of *C. concisus* DNA in clinical samples

The presence of *C. concisus* DNA was investigated directly in faeces, intestinal biopsy and saliva samples (Huq *et al.* 2014; Zhang *et al.* 2010; Zhang *et al.* 2009). Initially a primer set (C412F and C1288R) designed by Linton *et al.* (1996) to amplify the 16S rDNA gene (816 bp), was used as one step PCR to detect *C. concisus* from colonic biopsies (Zhang *et al.* 2009). Then, to identify *C. concisus*, the PCR product was sequenced and aligned to published sequences (Zhang *et al.* 2009). Following that, Man *et al.* (2010b) developed a specific nested PCR to detect *C. concisus* in faecal specimens also targeting the 16S rDNA gene. In the first step PCR, the primer set used was C412F and C1288R while in the second

step PCR a new primer set (ConcisusF and ConcisusR) was designed to specifically amplify 560 bp of *C. concisus* 16S rDNA gene. This nested PCR has been used for *C. concisus* DNA detection in other human clinical specimens including intestinal biopsies and saliva (Mahendran *et al.* 2011; Man *et al.* 2010b; Mukhopadhya *et al.* 2011; Zhang *et al.* 2010).

Recently, Huq *et al.* (2014) developed a multiplex PCR (m-PCR) to detect *C. concisus* and other members of *Campylobacter* in faecal samples, based on the size of PCR product. However, as *C. concisus* is present in very low numbers in intestinal samples, using the nested PCR could be more sensitive than m-PCR.

1.8.3.2 Molecular confirmation of *C. concisus* isolates

The first specific PCR used for *C. concisus* identification was developed by Bastyns *et al.* (1995) using the primer set MUC1 (a forward primer) and CON1/CON2 (reverse primers), which amplified the 23S rDNA region from all tested *C. concisus* isolates. Another primer set (Pcisus5-F and Pcisus6-R) to amplifies *gyrB* was developed by Matsheka *et al.* (2001). It was initially used to amplify DNA fragments (344 bp) obtained from a *C. concisus* genomic library and later shown to specifically amplify *C. concisus* (Huq *et al.* 2014; Istivan 2005; Matsheka *et al.* 2001).

1.9 Diversity of *C. concisus* strains

There is no standard classification method to fully address the diversity of *C. concisus*. The unavailability of standard typing methods has led to limitations in our understanding of the transmission, natural habitat and virulence of *C. concisus* (Lastovica 2006). Typing of *C. concisus* could determine whether isolates obtained from diarrhoeic patients differ from those colonising healthy individuals (Aabenhus *et al.* 2005a; Engberg *et al.* 2005; Macuch *et al.* 2000; Newell 2005).

1.9.1 Typing by protein profiling technique

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts has been applied successfully to distinguish *C. concisus* from other small oral bacteria with very similar characteristics to *C. concisus*, such as *C. mucosalis*, other species of Gram-negative rods, as well as non-pigmenting and asaccharolytic bacteria (Penner 1988; Tanner 1986). Other studies have used SDS-PAGE analysis of protein patterns to identify clusters within *C. concisus;* and the strains were classified into 2-5 clusters (Engberg *et al.* 2005; Istivan *et al.* 2004). Therefore, the protein profiling technique could be discriminative for *C. concisus* isolates, but the discriminative power might be improved if combined with another typing technique such as genotyping. It should also be noted that protein profiles of *C. concisus* isolates from IBD patients and healthy controls were indistinguishable in one study (Ismail *et al.* 2012).

1.9.2 Genomic typing by PCR and other techniques

There is no documented standard technique for genotyping *C. concisus* isolates. The first genetic method to type *C. concisus* was DNA-DNA hybridization (Vandamme *et al.* 1989). The genetic diversity was later confirmed by analysis of 100 *C. concisus* isolates using randomly amplified polymorphic DNA (RAPD) (Matsheka *et al.* 2006; Van Etterijck *et al.* 1996). Another approach used to type *C. concisus* was amplification of 23S rDNA (section 1.8.3.2; Bastyns *et al.* (1995)) using the specific primer set MUC1 and CON1 or MUC1 and CON2 (Aabenhus *et al.* 2005a; Istivan *et al.* 2004). In this system, isolates amplified by MUC1 and CON1 were assigned to genomospecies A, while those amplified by MUC1 and CON2 were designated genomospecies B (Aabenhus *et al.* 2005a; Istivan 2005; Istivan *et al.* 2004; Kalischuk *et al.* 2011). It was noted that there are six base pairs difference between the binding site of primers CON1 and CON2 on the genomes of these two genomospecies.

Through this study, genomospecies A and B will be used to refer the two genomospecies (A and B) generated by amplifying 23S rDNA using MUC1 and CON1/CON2.

Multilocus sequence typing (MLST) was also applied to 70 oral and intestinal *C. concisus* isolates from eight patients with IBD and six healthy controls (Figure 1.3) (Ismail *et al.* 2013). Subsequently, the neighbour-joining tree divided these isolates into 26 types (including *C. concisus* 13826) and two main groups (Figure 1.3). Most isolates (87.5%) in cluster 1 were from IBD patients compared with only 28.6% in cluster 2 (P <0.05). It was also found that all of the invasive *C. concisus* isolates were localised in cluster 1 (Ismail *et al.* 2013). Two main groups were also demonstrated by MLST using a different set of housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *ilvD*, and *pgm*), applied to 60 *C. concisus* faecal isolates (Miller *et al.* 2012). However in both of these studies, it was not clear whether the two main groups correlated with genomospecies A and B.

Pulsed field gel electrophoresis (PFGE) also indicated the diversity of *C. concisus* isolates and assigned them into two main groups according to the source of the isolates (faeces and the oral cavity), (Matsheka *et al.* 2002). In addition, *C. concisus* faecal isolates were allocated into four groups using the amplified fragment length polymorphism analysis technique (AFLP) (Aabenhus *et al.* 2005a; Kalischuk *et al.* 2011). Groups 1 (22 isolates) and 2 (32 isolates) matched with *C. concisus* genomospecies A and B (based on 23S rDNA PCR), while groups 3 (one isolate) and 4 (five isolates) were considered as additional genomospecies as they could not be amplified by 23S rDNA PCR (Aabenhus *et al.* 2005a). Group 2 isolates were found to be significantly more predominant in immunocompetent patients, in patients with chronic diarrhoea, bloody diarrhoea or with fever than group 1 isolates. Group 3 isolate was from an immunocompetent patient, while group 4 isolates were from immunodeficient patients with organ transplants or haematological malignancies (Aabenhus *et al.* 2005a).

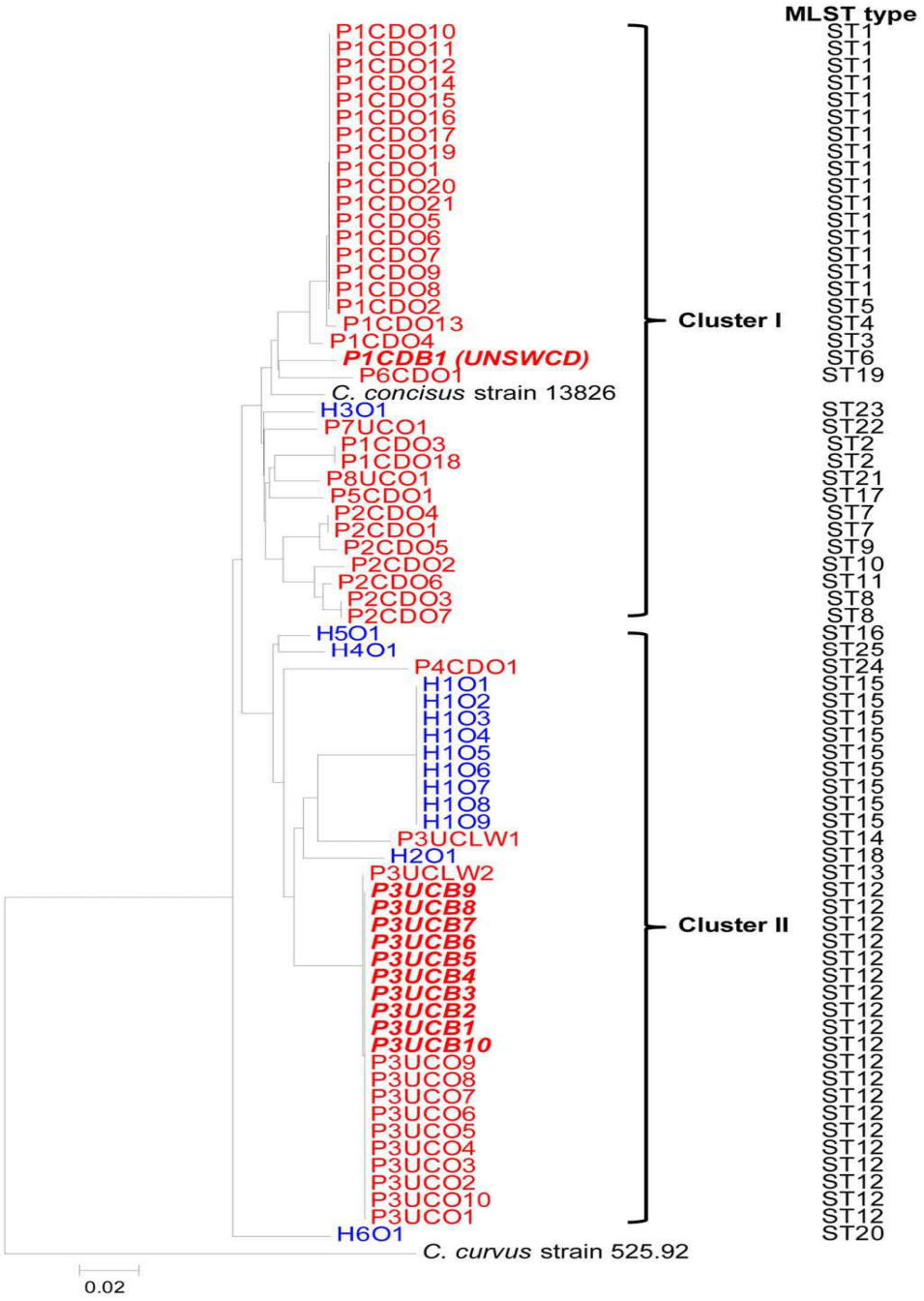


Figure 1.3: MLST analysis targeting six housekeeping genes (*asd, aspA, atpA, glnA, pgi* and *tkt*) of 60 *C. concisus* isolates from oral and enteric samples. The gene sequences were analysed by neighbour-joining tree. Red: *C. concisus* isolates from patients with IBD. Blue: *C. concisus* isolates from healthy individuals. The two main groups are depicted in roman numerals. This Figure was adapted from Ismail *et al.* (2012).

1.9.3 Typing by denaturing gradient gel electrophoresis (DGGE)

The PCR-DGGE technique was initially used to evaluate the microbial diversity in complex environments (Petersen *et al.* 2007). In environmental microbiology applications, universal primers are designed to target the 16S rDNA gene for the detection of mixed bacterial communities and differentiation of bacterial species (Petersen *et al.* 2007). The PCR product is separated by polyacrylamide gel electrophoresis based on the use of different melting temperatures and its mobility in gradient denaturation of formamide and urea (Fischer *et al.* 1983). A study conducted on DNA extracted from human saliva using PCR-DGGE to detect *Epsilobacteria* (Campylobacters, Helicobacters and Arcobacters and related bacteria) found the three reference strains of *C. concisus* fell into two different DGGE profile groups (Petersen *et al.* 2007). However, Cornelius *et al.* (2012) found *C. concisus* isolates assigned into only one DGGE profile group. The authors suggested that PCR-DGGE can be a useful tool to be used for a direct detection of *Epsilobacteria*. The application of DGGE to 21 *C. jejuni* isolates and one *C. coli* isolate using primer sets targeting the flagellin gene identified nine different groups (Najdenski *et al.* 2008).

1.9.4 Full genome sequencing of *C. concisus*

At the commencement of this study, *C. concisus* 13826 (also known *C. concisus* BAA-1457) was the only the strain to be completely sequenced (Table 1.3) (Fouts *et al.* 2007). Following that, other *C. concisus* strains were sequenced and are available in the NCBI data-base site as whole-genome shotgun contigs (WGS) (Deshpande *et al.* 2011; Deshpande *et al.* 2013) (Table 1.3). The sequencing of *C. concisus* revealed that some genes might not exist in all *C. concisus* strains. Consequently intensive genetic studies based on sequence comparisons would improve our understanding on the bacterial virulence and epidemiology.

Table 1.3: *C. concisus* strains that have been sequenced (whole-genome).

***C. concisus* strains**	**Sample type**	**Disease**	**Age**	**NCBI accession**	**Genome size (Mbp)**	**Year of sequencing**
13826	Faeces	Acute gastroenteritis	-	CP000792.1	2.05	Fouts *et al.* (2007)
UNSWCD	Intestinal biopsy	Crohn's disease	Child	AENQ00000000	1.81	Deshpande *et al.* (2011)
UNSW1	Intestinal biopsy	Chronic gastroenteritis	Child	ANNF00000000	1.94	Deshpande *et al.* (2013)
UNSW2	Intestinal biopsy	Crohn's disease	Child	ANNJ00000000	2.01	Deshpande *et al.* (2013)
UNSW3	Intestinal biopsy	Crohn's disease	Child	ANNE00000000	1.91	Deshpande *et al.* (2013)
UNSWCS	Faeces	Acute gastroenteritis	Child	ANNG00000000	2.11	Deshpande *et al.* (2013)
ATCC 51561	Faeces	None	Adult	ANNH00000000	1.99	Deshpande *et al.* (2013)
ATCC 51562	Faeces	Acute gastroenteritis	Child	ANNI00000000	1.84	Deshpande *et al.* (2013)

In summary, these studies show that *C. concisus* is genetically a diverse species, but the extent of the differences between strains remains largely unknown. There appear to be two main genomospecies (A and B), as demonstrated by PCR of 23S rDNA; however, these two genomospecies may not directly correspond to the two groups defined by PFGE, MLST and RAPD. These techniques, together with AFLP and whole genome sequencing demonstrate the diversity of *C. concisus*. Therefore, it is possible that certain groups could be more likely to be associated with clinical disease than others. From the reviewed studies, *C. concisus* strains should be assigned to at least two main groups in order to start generating a standard genetic profiling scheme for *C. concisus*.

1.10 Virulence factors in *C. concisus*

The number of studies investigating the potential pathogenic role of *C. concisus* has increased in the past three years possibly due to suggestions that *C. concisus* might have a potential association with IBD. Several putative virulence factors have been investigated including the capability for invasion of intestinal cell lines, haemolysin production and several other virulence mechanisms.

1.10.1 Invasion of *C. concisus* into host cells

The ability of *C. concisus* to invade and adhere to HEp-2 cells was investigated by Russell *et al.* (1998). Four faecal *C. concisus* isolates were 4-100-fold more invasive under these conditions than *C. jejuni* or *C. coli*. However *C. concisus* isolates differ in their ability to adhere to and invade human intestinal cell lines (Caco2 & LS174T), possibly due to the possession of a plasmid harbouring virulence factors (Kaakoush *et al.* 2011b; Kalischuk *et al.* 2011; Man *et al.* 2010a; Nielsen *et al.* 2011b). Importantly, the capability of *C. concisus* isolates from biopsies of children with chronic intestinal diseases to invade host cells was

significantly higher (500-fold) than for isolates from children with acute intestinal diseases or healthy controls (Kaakoush *et al.* 2011b). Moreover, Ismail *et al.* (2012) found that half of the patients with IBD were colonised by strains capable of invading intestinal cells (Caco2) in their oral cavities, while none of the strains isolated from the healthy controls were invasive. In contrast, Kalischuk *et al.* (2011) and Nielsen *et al.* (2011b) did not observe any differences in invasion, adherence or translocation of T84 and HT-29/B6 cells between *C. concisus* faecal isolates from patients with diarrhoea and healthy controls. These differences between studies could relate to the different cell lines used, the source of isolates or to the groups of isolates. Future adhesion and invasion studies using *C. concisus* groups may lead to finding a certain pathogenic group or subgroup or could indicate whether *C. concisus* is an opportunistic pathogen.

Furthermore, *C. concisus* isolates can stimulate pro-inflammatory cytokines of host cells *in vitro* (Kaakoush *et al.* 2011b; Kalischuk *et al.* 2011; Man *et al.* 2010a; Nielsen *et al.* 2011b). This stimulation depended on whether *C. concisus* isolates were invasive or adherent, for example, all adhesive/invasive isolates, unlike adhesive/non-invasive isolates up-regulated IL12 and induced interferon-γ during the infection of the host cells. The authors suggested that the *in vitro* response had significant similarities to the immune response observed in the mucosa of CD patients (Kaakoush *et al.* 2011b). Recently, Burgos-Portugal *et al.* (2014) reported that inhibition of autophagy increased up to 2-4 fold as the numbers of *C. concisus* in host cells (Caco-2) increased, leading to a significant reduction in *C. concisus* clearance. They concluded that *C. concisus* could manipulate the host cell autophagy process to enhance its survival (Burgos-Portugal *et al.* 2014).

1.10.1.1 Mechanism of *C. concisus* adherence and invasion

Based on scanning electron microscopy images, the initial contact between *C. concisus* and the host cells appears to be with the flagellum. The adherence mechanism was suggested to be an interaction between *C. concisus* cells and intestinal mucosal cells with the possibility of biofilm formation (Kaakoush *et al.* 2011b) as *C. concisus* ATCC 33237 is able to aggregate and grow within biofilms (Gunther *et al.* 2009). Following attachment, the bacterial cells penetrate the epithelial cell membrane. This process is mediated by secretion of a fibronectin binding protein at the host cell outer membrane (Man *et al.* 2010a), as shown in Figure 1.4. This mechanism was suggested to be similar to *C. jejuni* invasion (Kaakoush *et al.* 2011b).

1.10.2 Presence of putative virulence genes in *C. concisus*

Only limited studies have investigated *C. concisus* virulence genes. As whole genome sequencing has shown that *C. concisus* strains are genetically heterogeneous and differ in the number of genes they possess, studying putative virulence genes could improve our understanding of any role in virulence and in the taxonomy of this species. The following genes will be investigated in this study as they have been reported to be putative virulence factors in other pathogenic *Campylobacter* spp.: *cjaC, cjaA, dnaJ* and *zot.* In *C. jejuni,* the *cjaC* and *cjaA* genes encode immunodominant proteins and may have a role in the bacterial virulence status (Laniewski *et al.* 2014; Muller *et al.* 2005; Wyszynska *et al.* 2004). The *cjaA* gene also involved in increasing bacterial resistance to β-lactam antibiotics (Mansell *et al.* 2013). The *dnaJ* is a stress gene and it is one of virulence-associated genes in *C. jejuni* (Bui *et al.* 2012; Konkel *et al.* 1998). The *zot* gene encodes zonula occludens toxin and it has been used by several pathogenic bacteria (Fasano *et al.* 1995).

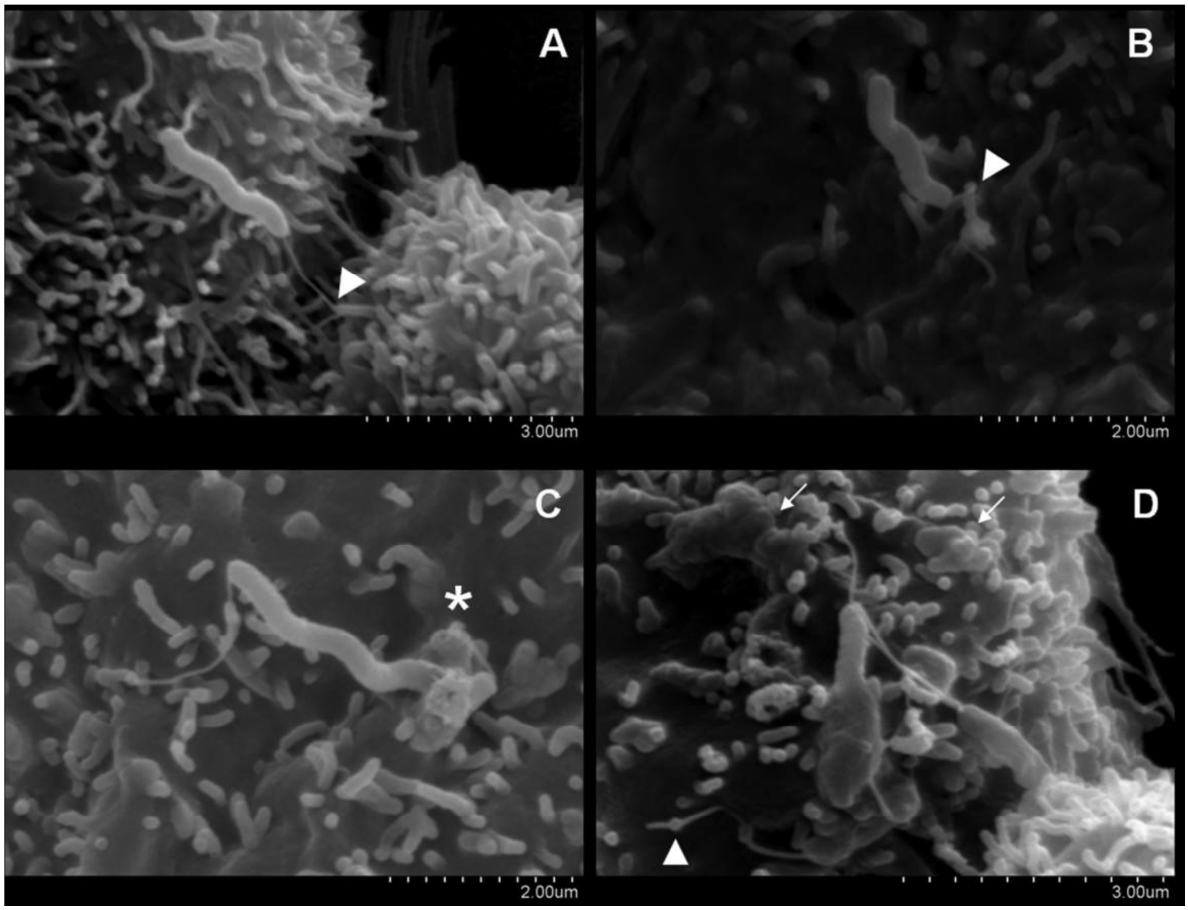


Figure 1.4: Adherence and invasion of *C. concisus* UNSWCD. A) The initial contact and attachment of *C. concisus* into the host cell (Caco-2) by interaction of the bacterial polar flagellum and the microvillus tip (triangles). B) The bacterial flagellum folds around the microvillus (triangle). C) *C. concisus* induces and performs a membrane ruffling-like effect (*). D) Half of the *C. concisus* cell appears to penetrate and internalise the membrane of the host cell, causing a membrane surface protrusion, and half of its flagellum appears outside the host cell. The host cell appears to be infected by more than one bacterial cell as the host cell displays irregularities and a rough textured membrane on the cell due to protrusions induced during the bacterial invasion (arrows). These images were adapted from Man *et al.* (2010a).

1.10.2.1 The *cjaC* and *cjaA* genes

The *cjaC* (trap-type mannitol/chloroaromatic compound or *C. jejuni* antigen C) and *cjaA* genes (*C. jejuni* antigen A) encode secreted proteins, which are amongst the ABC components of outer membrane proteins (Pawelec *et al.* 1998; Wyszynska *et al.* 2004). The ABC system is a complex of several components that are implicated in the cell membrane transport system to control solute movements throughout biological membranes of cells. The ABC export systems have been found in all microorganisms that have been studied (Pawelec *et al.* 1998; Saier 1994; Tam *et al.* 1993). Approximately 100 systems have been determined to date (Fath *et al.* 1993; Higgins 1995; Tam *et al.* 1993). In Gram-negative bacteria, ABC transporting systems consist of soluble proteins generally found in the periplasm; whereas in Gram-positive bacteria, these proteins are present on the external surface of the cell membrane as anchored lipoproteins.

The cellular location of the CjaC and CjaA proteins depends on the hosts. These proteins are present in the periplasmic space in *E. coli*, while they are found in the inner membrane as anchored proteins in *C. jejuni* and *C. coli* (Wyszynska *et al.* 2007; Wyszynska *et al.* 2008). In campylobacters, the CjaC and CjaA proteins may be responsible for histidine and cysteine up-take, respectively (Muller *et al.* 2005; Wyszynska *et al.* 2004; Wyszynska *et al.* 2007). They might be vital for campylobacters, which are asaccharolytic microorganisms that use amino acids as an alternative carbon source (Wyszynska *et al.* 2007). Although bioinformatic predictions for *C. concisus* UNSWCD showed that the CjaC and CjaA could be secreted proteins (Kaakoush *et al.* 2010), the cellular location and function of the corresponding genes has not been investigated. The CjaC and CjaA proteins in *C. concisus* are predicted to have a similar function as the corresponding genes in *C. jejuni* (Kaakoush *et al.* 2010).

Furthermore in other campylobacters, the CjaC and CjaA proteins are highly immunogenic surface proteins and are likely to play a substantial role in bacterial virulence (Muller *et al.*

2005; Wyszynska *et al.* 2004). The product of a cloned *cjaC* gene of *C. jejuni* $72D_z/92$ was highly immunogenic in rabbits (Pawelec *et al.* 1998) and was also suggested as a good vaccine candidate in chickens (Skirrow 1992). Moreover, the CjaA protein from *C. jejuni* was effective as a vaccine against different *Campylobacter* species (Clark *et al.* 2012; Wyszynska *et al.* 2004). In *C. concisus*, the CjaC and CjaA proteins were reported to show immunoreactivity against CD patients' sera (Kovach *et al.* 2011).

1.10.2.2 The *dnaJ* gene

The *dnaJ* gene encodes a stress protein, which belongs to Hsp-40 (heat shock protein 40 KD); a family of molecular chaperones that are produced by many bacterial species (Konkel *et al.* 1998). *DnaJ* is considered to be among virulence-associated genes (Li *et al.* 2008) that may play a significant role in *C. jejuni* colonisation in chickens (Konkel *et al.* 1998) since mutant *dnaJ* strains grow slowly at 46°C and fail to colonise newly hatched Leghorn chickens (Konkel *et al.* 1998). In *C. concisus*, *dnaJ* has not been investigated but could be involved in colonising different areas of the human body such as the oral cavity, intestinal tract and faeces.

1.10.2.3 The *zot* gene

The ZoT protein, a tight junction toxin encoded by the *zot* gene that is expressed by virulent bacteria such as *Neisseria meningitides* and *Vibrio cholera* (Fasano *et al.* 1995). Recently, a related toxin has been detected in *C. concisus* (Kaakoush *et al.* 2010), *C. ureolyticus* (Bullman *et al.* 2013), but not in other *Campylobacter* spp. (Kaakoush *et al.* 2010). Alignment of the translated protein sequence from *C. concisus* 13826 to similar proteins from *N. meningitidis* and *V. cholerae* showed four conserved domains suggesting that these could be linked to the ZoT toxin activity (Figure 1.5) (Kaakoush *et al.* 2010). *C. concisus* strains may have acquired the gene from these other pathogens by gene transfer (Kaakoush *et al.*

2010; Zhang *et al.* 2014). The gene transfer may occur by a specific species of a prophage containing the *zot* gene as the majority of *Campylobacter* spp. do not possess the gene. The function of ZoT is to enhance tissue permeability (Fasano *et al.* 1995) as its interaction with receptors on host cells results in intracellular signalling activation, which ultimately disassembles the intercellular tight junctions (Di Pierro *et al.* 2001). Therefore, it may play a role in *C. concisus* virulence (Kaakoush *et al.* 2010; Uzzau *et al.* 2000). In *C. concisus,* polymorphic forms of *zot* in which valine at position 270 is substituted with other amino acids may be linked to patients with active IBD (Mahendran *et al.* 2013).

1.10.3 Other putative toxins produced by *C. concisus*

Several toxins have been reported to be produced by *C. concisus* isolates. Initially, *C. concisus* isolates from children with gastroenteritis were shown to lyse human and animal erythrocytes (Istivan *et al.* 1998; Kalischuk *et al.* 2011). Extracted outer cell membrane proteins of *C. concisus* of both genomospecies A and B possess haemolytic phospholipase A2 activity, that caused stable vacuolating and cytolytic effects on ovarian cells of Chinese hamster (CHO) in tissue culture, providing strong suggestive evidence that *C. concisus* possess virulence mechanisms (Istivan *et al.* 2004; Istivan *et al.* 2008). Recently, cytotoxic activity by oral and faecal isolates of *C. concisus* to intestinal cell lines (HT-29/B6 and T84) was demonstrated by showing the release of high levels of lactate dehydrogenase (LDH) and induction of apoptotic DNA fragmentation (Kalischuk *et al.* 2011; Nielsen *et al.* 2011b).

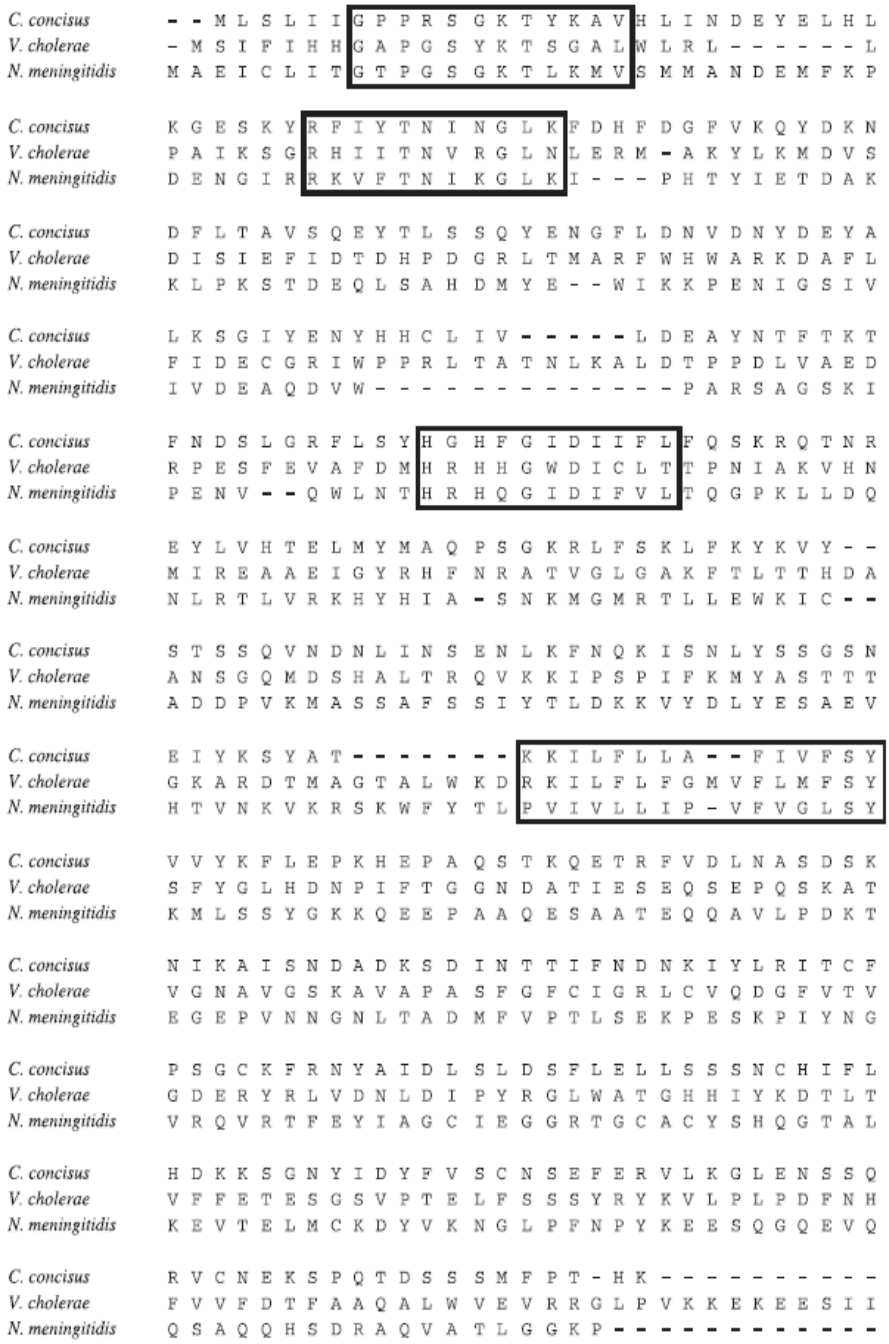


Figure 1.5: Alignment of ZoT toxin sequences from the genomes of *C. concisus* 13826, *V. cholerae* 86015 and *N. meningitidis* MC58. The sequences in boxes refer to the highly conserved domains (this Figure was adapted from Kaakoush *et al* (2010)).

1.11 *C. concisus* and biofilm formation

C. concisus isolates obtained from faeces (of gastroenteritis patients and a healthy control), colonic biopsies (of CD patients) and the oral cavity (of a patients with periodontitis) were able to form biofilms on all tested surfaces (stainless steel, glass and polystyrene plastic) with no significant differences in biofilms formation between these isolates (Figure 1.6) (Gunther *et al.* 2009; Lavrencic *et al.* 2012). The biofilms formed by *C. concisus* were described to have higher consistency compared to other 16 tested *Campylobacter* spp. (Gunther *et al.* 2009).

These studies demonstrated another possible virulence factor of *C. concisus*. The bacterium could produce biofilm in the human gut leading to long term residence in a protected environment in the intestinal tract. It is most likely that biofilms help bacteria to survive in the gut by protection from the immune response of the host cell and antibacterial agents (Donlan 2002).

1.12 Motility of *C. concisus*

Motility can facilitate bacterial virulence since highly motile bacterial cells are able to swim through the protective barrier of the intestinal mucosa, allowing direct contact with host cells (Haiko *et al.* 2013). Intestinal *C. concisus* isolates display two distinct types of motility (Lavrencic *et al.* 2012) and attracted by several chemicals including formate and mucin (Kaakoush *et al.* 2011b; Paster *et al.* 1986). However, the authors did not correlate the two types of motility to the genetic diversity of *C. concisus* isolates (Lavrencic *et al.* 2012).

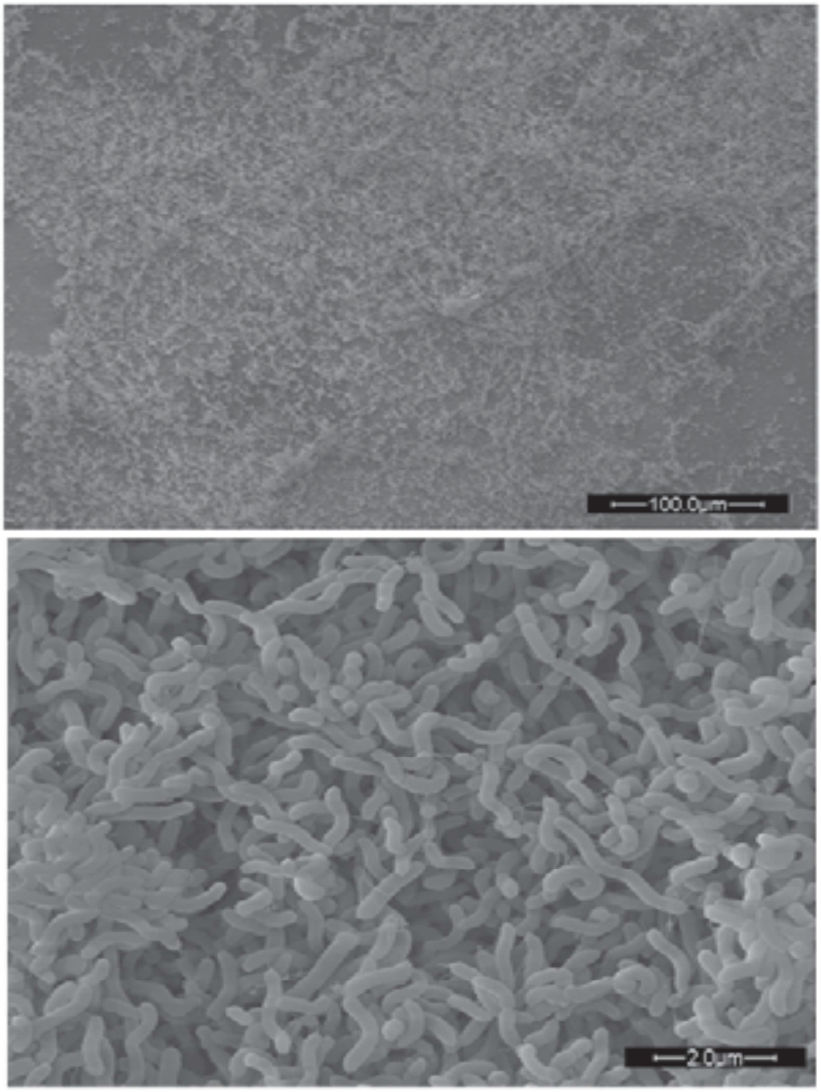


Figure 1.6: Scanning electron images for *C. concisus* (ATCC 33237) forming biofilms on glass. These images were adapted from Gunther *et al.* (2009).

1.13 *C. concisus* and antimicrobial susceptibility

The effects of antimicrobial agents on *C. concisus* are still not well studied, possibly due to the lack of standard validated techniques for use in the diagnostic laboratory. The disc diffusion method could be unsuitable for these slow growing bacteria (Istivan *et al.* 2010). Moreover, it is unclear which medium is most useful for antibiotic susceptibly testing. As most studies have used HBA to grow *C. concisus*, incorporating the antimicrobial agents into HBA would seem logical.

In most of the reviewed studies, *C. concisus* was found to be susceptible to the majority of antimicrobial agents including erythromycin, ciprofloxacin, tetracycline, ampicillin, clindamycin, azithromycin, gentamicin, chloramphenicol, cephalothin and ceftriaxone (Aabenhus *et al.* 2005b; Pruckler *et al.* 2002; Vandenberg *et al.* 2006), while it was mainly resistant to nalidixic acid, rifampin, vancomycin and bacitracin (Aabenhus *et al.* 2005a; Istivan 2005; Pruckler *et al.* 2002; Tanner *et al.* 1981). Remarkably, Moore *et al.* (2006) studied the change in the resistance state of 457 *C. concisus* isolates obtained between 1998-2006, against several antimicrobial agents and it was found that resistance increased by 22% for nalidixic acid, 16.9% for erythromycin, 11.1% for ciprofloxacin and 0% for ceftriaxone.

1.13.1 Treatment of *C. concisus* infections with antimicrobial agents

Most campylobacter infections are self-limited and treatment with antimicrobial agents is usually not required, particularly for gastroenteritis. The role of treatment with antimicrobial agents for other infections possibly by *C. concisus* is still not well addressed (Fitzgerald *et al.* 2008); however, infections in immunocompromised, elderly and immune deficient patients should be treated with antimicrobial agents (Hess *et al.* 2012; Zhang *et al.* 2009). Antimicrobial agents that are effective against infections due to other campylobacters can be effective for the treatment of *C. concisus*, in particular the antibiotics that inhibit RNA

synthesis, for example rifaximin (Guslandi *et al.* 2009). Erythromycin was suggested to be the antibiotic of first choice to treat intestinal campylobacteriosis caused by *C. concisus* (Vandenberg *et al.* 2006). Other suitable antimicrobial agents are ciprofloxacin, ampicillin and ceftriaxone.

In summary, the main reservoir of *C. concisus* is the human oral cavity. The organism is fastidious and its taxonomy is not well established. It is clear from several studies that the species is highly diverse, with some strains having the role of colonisers and others as possible pathogens. *C. concisus* has been isolated from cases of periodontitis/gingivitis, gastroenteritis and recently from intestinal biopsies of patients with IBD, but the role of *C. concisus* as causative agents for these infections is still unclear. Putative virulence factors include invasiveness, toxin production, haemolysin activity, and the ability to grow as biofilms. Most of the reviewed studies have urged intensive work to expand our knowledge of this organism in order to obtain clear views on its role in common diseases including gastroenteritis, infections of the oral cavity infections and IBD.

1.14 Project aims

This study investigated the role and prevalence of *C. concisus* as a possible pathogen in patients with enteric diseases by achieving these objectives:

> **Objective 1:** Type a collection of *C. concisus* isolates from carriage and disease states by DGGE, presence and variation of virulence genes (*cjaC, cjaC, dnaJ* and *zot*), and bioinformatics analysis in order to confirm the diversity of the species and detect the association of particular types with disease status.

Objective 2: Investigate the capability of *C. concisus* oral and faecal isolates to invade intestinal epithelial cell line (INT407) and examine the expression of the possible virulence genes (*cjaC, cjaC, dnaJ* and *zot*) to evaluate the virulence state of the isolates obtained from different sites.

Objective 3: Detect the prevalence of *C. concisus* in clinical samples (intestinal biopsies, faeces and gum swabs) collected from patients with IBD and control participants by culture and molecular techniques.

Objective 4: Develop nested PCRs to detect the possible involvement of genomospecies B and *zot* in IBD patients.

Objective 5: Use SDS-PAGE analysis to evaluate the similarity between *C. concisus* isolates obtained from different sites (intestine and mouth) of the same patient in order to investigate a possible association of *C. concisus* in patients with IBD.

Chapter 2 : General materials and methods

2.1 General procedures

Chemicals used in this study were analytical and molecular grade reagents unless otherwise stated. The solutions used were prepared in deionised water (dH_2O) filtered by a Millipore Milli-Q® water system (Liquipure) except where otherwise itemised, and stored at room temperature except where otherwise mentioned. Small volumes of the solutions were dispensed with micropipettes of Finnpipette (Pathtech Pty Ltd., Australia) using the range of the following volumes: 200 µl - 1 ml, 20 µl - 200 µl, 5 µl - 50 µl, 0.5 µl - 10 µl and 0.1 µl - 2 µl. All reusable plasticware and glassware was washed and cleaned by Pyroneg detergent, then rinsed in tap water and finally with dH_2O. Sterilisation of glassware, autoclavable consumables (micropipette tips, 1.5 ml tubes, toothpicks and PCR tubes), media, used bacterial solutions like dH_2O and PBS was autoclaved at 121°C for 20 min unless otherwise indicated.

2.2 General materials and equipment

2.2.1. General materials

Reagent	Supplier
Acetic acid	Sigma-Aldrich Pty. Ltd., Australia
Acetone	Sigma-Aldrich Pty. Ltd., Australia
Acrylamide, 40% (W/V) solution	Astral Scientific Pty. Ltd, Australia
Agarose (DNA grade)	Bioline, Australia
Albumin, bovine serum	Sigma-Aldrich Pty. Ltd., Australia
Ammonium acetate	Ajax Chemicals Ltd, Australia
Ammonium chloride	BDH Chemicals, Australia
Ammonium hydroxide	Sigma-Aldrich Pty. Ltd., Australia
Ammonium persulfate	Bio-Rad Laboratories, Australia
Ammonium sulphate	Sigma-Aldrich Pty. Ltd., Australia
Bacteriological tryptone	Cell BioSciences, Australia
Brain-Heart infusion broth	Oxoid, Australia
Brilliant Blue G (for Coomassie blue)	Sigma-Aldrich Pty. Ltd., Australia
Bromo-phenol blue	Sigma-Aldrich Pty. Ltd., Australia
CampyGen Sachet (Gas Pack)	Oxoid, Australia

Columbia agar base	Cell BioSciences, Australia
Columbia Broth	Cell BioSciences, Australia
Coomassie brilliant blue	Coomassie Brilliant Blue R-250
Brilliant blue G	Sigma-Aldrich Pty. Ltd., Australia
Copper sulphate	Merck, Australia
Dimethylsulphoxide (DMSO)	Merck, Australia
Dulbecco's Modified Eagle's Medium (DMEM)	Life technologies, Australia
Ethanol	Merck, Australia
Ethidium bromide	Sigma-Aldrich Pty. Ltd., Australia
EDTA (Ethylene diamine tetra acetic acid)	Merck, Australia
Fetal bovine serum (FBS)	Bovogen serumBiologicals, Australia
Ficoll-400 Reagent	Merck, Australia
Folin-Ciocalteau reagent	Sigma-Aldrich Pty. Ltd., Australia
Formaldehyde	Merck, Australia
Formamide	Merck, Australia
Gentamicin sulfate	Sigma-Aldrich Pty., Ltd, Australia
Glycerol	Merck, Australia
Glycine	BDH Chemicals, Australia

Ham's F-12	Life technologies, Australia
HEPES buffer (1M) (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid)	Thermofisher, Australia
Horse Blood (Defibrinated)	Oxoid, Australia
Hydrochloric acid (32%)	Merck, Australia
Hydrogen peroxide (30 %)	BDH Chemicals, Australia
Lambda DNA (50 µg)	Biolabs, Australia
Immersion oil (Gurr)	BDH Chemicals, Australia
Isolate II Genomic DNA kit	Bioline, Australia
Isolate RNA Mini kit	Bioline, Australia
Isolate II PCR and Gel Kit	Bioline, Australia
Isopropanol	Merck, Australia
Lysozyme	Astral Scientific Pty. Ltd, Australia
Magnesium chloride (Hexahydrate)	BDH Chemicals, Australia
Magnesium Sulfate	BDH Chemicals, Australia
β-Mercaptoethanol	Sigma-Aldrich Pty. Ltd., Australia
Methanol	BDH Chemicals, Australia
Microaerophilic gas mixture	Linde Gas, Australia
MyTaq DNA Polymerase	Bioline, Australia
Orange G	Merck, Australia
ParaFilm tape	InterPath, Australia
Petri dish	VWR international, Australia
Phosphate buffer saline (PBS) tablets	Astral Scientific Pty. Ltd, Australia
Potassium chloride	BDH Chemicals, Australia

Protein Prestained standards marker	Bio-Rad, Australia
*Pst*I (100 U/µL).	Biolabs, Australia
SensiFAST^{TM} SYBR No-ROX' kit	Biolabs, Australia
Skim milk	Coles, Australia
Sodium acetate	BDH Chemicals, Australia
Sodium bicarbonate	Cytosystems Pty Ltd, Australia
Sodium Carbonate	Merck, Australia
Sodium chloride	Merck, Australia
Sodium dodecyl sulfate (SDS)	Merck, Australia
Sodium hydroxide	Merck, Australia
Sodium Potassium Tartrate	Merck, Australia
Sulphuric acid	BDH Chemicals, Australia
TEMED (N,N,N',N'tetramethylethylenediamine)	Bio-Rad, Australia
Tetro cDNA Synthesis kit	Bioline, Australia
Tissue culture flask (25 cm^2, 75 cm^2)	VWR, Australia
Tris-HCl	Merck, Australia
Triton-X-100	Sigma-Aldrich Pty., Ltd, Australia
Trypan blue	Sigma-Aldrich Pty., Ltd, Australia
Trypsin – EDTA	Life Technology, Australia
Tryptone	Cell BioSciences, Australia
Turbo DNA-free^{TM} kit	Ambion, Life technologies, Australia
Ultrapure water	Bioline, Australia

UltraPure Tris-base	Sigma-Aldrich Pty. Ltd., Australia
Urea	BDH Chemicals, Australia
Wizard Genomic DNA Purification Kit	Promega, Australia
Yeast extract	Cell BioSciences, Australia

2.2.2. General equipment

Equipment	**Supplier**
Anaerobic jars: 2.5 L, 3.5 L and 10 L	Oxoid, Australia, Don Whitely, Australia
Analytical balance	Sartorius GMBH, Australia
Balance (0.1-500 g)	U-Lab, Australia
Bench top centrifuge	Eppendorf, Australia
Biological Safety Cabinet Class II	LAF Technologies, Australia
Cell counting chamber	Life Technology, Australia
Cell Counter	Life technology. Australia
Cellulose Acetate Filter (0.65 µm)	Sartorius GMBH, Australia
Centrifuge tubes:	
- 1.5 ml Eppendorf centrifuge tube.	VWR, Australia
- 10 ml centrifuge tube	VWR, Australia
- 15 ml centrifuge tube	VWR, Australia
- 50 ml centrifuge tube	VWR, Australia

Copan Eswab	Copan Diagnostics, Inc**,** USA
Cover slips	Mediglass, Australia
Cryovials (1.8 ml)	Mcfarlane Medical, Australia
Cuvette	Eppendorf, Australia.
DNA Thermocycler (for PCR)	G-Storm, England
Electrophoresis Power Supply	
(A) PowerPac Basic	Bio-Rad, Australia
Electrophoresis Units:	
(1) DNA:	
- Mini Gel	Bio-Rad, Australia
- Midi Gel	Bio-Rad, Australia
(2) Protein:	
- Mini Protein II gel system	Bio-Rad, Australia
Filters: syringe Filters (0.22 µm)	Sarstedt, Germany
GELDOC system	Bio-Rad, USA
Tissue culture (5% CO_2) incubator	Forma Scientific, USA
Loops: normal plastic & L-shape	Interpath, Australia
Microscopes:	
- Light microscope	Olympus optical, Japan
- Inverted Optical ix71	Olympus optical, Japan
pH meter	Metrohm, Swiss

Plate (24 and 96 wells)	VWR, Australia
Rotor-Gene Q (Real-Time PCR)	Qiagen, Australia
Centrifuges:	
- Microcentrifuge	Zentrifugen, Germany
- High-Speed centrifuge	Thermofisher, Australia
Scanner (SDS-PAGE imaging)	Canon, Australia
Shaker (with incubation service)	Bioline Global, Australia
Slides	LivingStone, Australia
Spectrophotometer (OD_{600})	Eppendorf, Australia
SpectroStar Omega Reader (ELISA reader for Protein Concentration)	BMG-LabTech, Australia
Syringe (1 ml, 5 ml, 10 ml, 20 ml, 50 ml)	LivingStone International, Australia
Syringe needle (18G)	Terumo Pty, Ltd., Australia
Tips (Sterile filtered 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl)	VWR, Australia
Tips (10 µl, 200 µl and 1000 µl)	VWR, Australia
Vortex mixer	Ratek Instruments, Australia
Water Bath	Ratek Instruments, Australia
Weight Trays	Mirella Research, Australia

2.3 Bacteriological media

2.3.1 Brucella broth

Brucella broth powder (2% w/v) was dissolved in dH_2O and then sterilised by autoclaving under standard conditions (121°C for 15 min under 1.05 kg/cm^2 pressure).

2.3.2 Brain heart infusion broth

Brain heart infusion broth powder (3.7% w/v) was dissolved in dH_2O and sterilised by autoclaving under standard conditions. It was then cooled down until approximately 55°C and aliquoted into 50 ml plastic tubes prior to storing at 4-8°C.

2.3.3 Columbia broth

Columbia broth base powder (3.5% w/v) was dissolved in dH_2O and sterilised by autoclaving under standard conditions. It was cooled until approximately 27°C (room temperature) and aliquoted into 50 ml plastic tubes prior to storing at 4-8°C.

2.3.4 Columbia agar (CA)

Columbia agar base (Acumedia, USA) powder (4.1% w/v) was dissolved in dH_2O and sterilised by autoclaving using standard conditions. Then, it was cooled until approximately 55°C. Following that, around 25 ml of the medium were poured into 90 mm sterile Petri dishes. The plates were left to solidify at room temperature for two hours prior to storage at 4-8°C.

2.3.5 Horse blood agar (HBA)

Columbia agar base powder (4.1% w/v) was dissolved in dH_2O and sterilised by autoclaving using standard conditions. Then, it was cooled down until approximately 55°C. Defibrinated

horse blood (5% v/v) (Oxoid, Australia) was added to the medium, mixed thoroughly and approximately 25 ml of the mixture was poured into 90 mm sterile Petri dishes. The plates were left to solidify at room temperature for 2 h. The plates were stored at 4-8°C.

2.4 Bacterial isolates and growth conditions

2.4.1 Bacterial isolates

A total of thirty-three *C. concisus* isolates and three *Campylobacter* reference strains were recruited in this study. *C. concisus* isolates were obtained from two sources. The first source was a collection of 15 *C. concisus* isolates and two *C. concisus* reference strains from RMIT University bacterial stock collection as listed in Table 2.1. In this collection, the isolates were obtained from children who suffered from slight to severe bloody diarrhoea attending the Royal Children's Hospital (RCH), Victoria, Australia (Istivan et al. 2004). The second was a collection of 16 oral isolates obtained from healthy volunteers (adults and children at RMIT University, Melbourne, Australia) (Table 2.1). In this study the ethics approval for the second collection was made and granted by RMIT University Human Research Ethics Committee (HREC) (Ethics no. 57/13).

The oral isolates obtained for this study and were identified as described by Tanner *et al.* (1981) and Vandamme *et al.* (2005). The isolation of *C. concisus* was performed by collecting gum swabs from the participants. Suspected colonies grown on HBA were identified by morphological characterisation as mentioned in section 2.4.3 to confirm the identity of *C. concisus* isolates in Table 2.1.

Table 2.1: *C. concisus* isolates and *Campylobacter* reference strains used in this study.

Strain no	Source of the strain	Clinical manifestation	Age and sex	classification	Isolate collected location
RCH3	Faeces	3 days diarrhoea & vomiting	1 y, male	*C. concisus* (A)	RCH, Australia
RCH4	Faeces	5 days diarrhoea & vomiting	1 y, female	*C. concisus* (A)	RCH, Australia
RCH5	Faeces	5 days diarrhoea	5 m, female	*C. concisus* (A)	RCH, Australia
RCH6	Faeces	3 days diarrhoea	2 y, male	*C. concisus* (B)	RCH, Australia
RCH7	Faeces	7 days gastroenteritis & vomiting	16 m, male	*C. concisus* (A)	RCH, Australia
RCH9	Faeces	7 days diarrhoea & fever	2 y, male	*C. concisus* (A)	RCH, Australia
RCH11	Faeces	4 days diarrhoea	2 y, male	*C. concisus* (B)	RCH, Australia
RCH12	Faeces	20 days diarrhoea	30 m, male	*C. concisus* (A)	RCH, Australia
RCH15	Faeces	9 days gastroenteritis & vomiting	5 m, female	*C. concisus* (A)	RCH, Australia
RCH19	Faeces	5 days gastroenteritis & vomiting	2 y, female	*C. concisus* (A)	RCH, Australia
RCH20	Faeces	14 days gastroenteritis & vomiting	10 m, female	*C. concisus* (A)	RCH, Australia
RCH23	Faeces	gastroenteritis	< 15 y,	*C. concisus* (A)	RCH, Australia
RCH24	Faeces	gastroenteritis	< 15 y	*C. concisus* (A)	RCH, Australia
RCH25	Faeces	gastroenteritis	< 15 y	*C. concisus* (A)	RCH, Australia
RCH26	Faeces	gastroenteritis	< 15 y	*C. concisus* (A)	RCH, Australia
O1	Oral swab	healthy	41 y, male	*C. concisus* (A)	RMIT, Australia
O2	Oral swab	healthy	41 y, male	*C. concisus* (A)	RMIT, Australia
O3	Oral swab	healthy	41 y, male	*C. concisus* (A)	RMIT, Australia
O4	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O5	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O6	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O7	Oral swab	healthy	60 y, male	*C. concisus* (A)	RMIT, Australia
O8	Oral swab	healthy	3 y, female	*C. concisus* (B)	RMIT, Australia
O9	Oral swab	healthy	3 y, female	*C. concisus* (A)	RMIT, Australia
O10	Oral swab	healthy	27 y, female	*C. concisus* (A)	RMIT, Australia
O11	Oral swab	healthy	27 y, female	*C. concisus* (B)	RMIT, Australia
O12	Oral swab	healthy	5 y, male	*C. concisus* (A)	RMIT, Australia
O13	Oral swab	healthy	5 y, male	*C. concisus* (B)	RMIT, Australia
O14	Oral swab	healthy	27 y, male	*C. concisus* (A)	RMIT, Australia
O16	Oral swab	healthy	5 y, male	*C. concisus* (B)	RMIT, Australia
O17	Oral swab	healthy	5 y, male	*C. concisus* (A)	RMIT, Australia
O2	Oral swab	healthy	41 y, male	*C. concisus* (A)	RMIT, Australia
O3	Oral swab	healthy	41 y, male	*C. concisus* (A)	RMIT, Australia
O4	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O5	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O6	Oral swab	healthy	35 y, male	*C. concisus* (A)	RMIT, Australia
O7	Oral swab	healthy	60 y, male	*C. concisus* (A)	RMIT, Australia
O8	Oral swab	healthy	3 y, female	*C. concisus* (B)	RMIT, Australia
O9	Oral swab	healthy	3 y, female	*C. concisus* (A)	RMIT, Australia
O10	Oral swab	healthy	27 y, female	*C. concisus* (A)	RMIT, Australia
O11	Oral swab	healthy	27 y, female	*C. concisus* (B)	RMIT, Australia

O12	Oral swab	healthy	5 y, male	*C. concisus* (A)	RMIT, Australia
O13	Oral swab	healthy	5 y, male	*C. concisus* (B)	RMIT, Australia
O14	Oral swab	healthy	27 y, male	*C. concisus* (A)	RMIT, Australia
O16	Oral swab	healthy	5 y, male	*C. concisus* (B)	RMIT, Australia
O17	Oral swab	healthy	5 y, male	C. concisus (A)	RMIT, Australia
ATCC 51561^T	Faeces	healthy	24y, female	*C. concisus* (B)	CCUG 20034
ATCC 51562^T	Faeces	diarrhoea	7m, male	*C. concisus* (A)	CCUG 20700
81116	Human faeces	-	-	*C, jejuni*	UK, 1983
ATCC 43264^T	pig	-	-	*C. mucosalis*	CCUG 10771
NCTC 11366^T	Pig	-	-	*C. coli*	Belgium, 1980

ATCC 51561 and ATCC 51562: *C. concisus* reference strains. ATCC 43264: *C. mucosalis* reference strain. 81116: *C. jejuni* strain. NCTC 11366: *C. coli* reference strain. RCH: *C. concisus* isolates were collected previously from The Royal Children's Hospital, Victoria, Australia. O: the oral *C. concisus* isolates were obtained in this study. Y: years. m: months

2.4.2 Bacterial growth conditions

C. concisus was cultivated on HBA plates unless otherwise specified. The plates were incubated at 37ºC for 2-4 days under microaerobic conditions with a gas mixture consisting of 6% O_2, 8% CO_2, 6-7% H_2, and 80% N_2. All bacterial isolates used in this study were obtained from cultures no older than 2-3 passages.

2.4.3 Identification of *C. concisus*

The identification of *C. concisus* was initially by morphological characterisation of bacterial colonies. Suspected *C. concisus* colonies at 48 h that were small in size (0.5-1.0 mm in diameter), grey in colour, round in shape and convex with entire edge, were subjected to Gram-stain, oxidase test, catalase test, ability to grow in microaerobic conditions (section 2.4.2) on HBA, and then confirmed by molecular techniques.

2.4.4 Bacterial storage

Bacterial strains were grown on HBA plates for 2-4 days. The bacterial growth was harvested from the plate using a plastic loop and suspended in a cryovial containing ~1 ml tryptone skim milk. The bacterial suspension was stored at -80°C.

The preparation of tryptone skim milk was as following: Tris-HCl (157.6 mg) was dissolved in dH_2O (100 ml) to give a final concentration of 10 mM. Skim milk (10 % w/v) and tryptone (1 % w/v) were dissolved in the prepared solution with a final adjustment of pH at 7.5 using NaOH. The solution was gently mixed and each 10 ml aliquot was dispensed into a small glass container. The containers were then autoclaved at 109°C for 30 min before the containers were stored at 4-8°C.

2.5 Genomic bacterial DNA techniques

2.5.1 DNA extraction

Bacterial growth from an entire HBA plate (2-4 days old) was collected and suspended in 1 ml PBS. The suspension was centrifuged at 14000 x *g*. The bacterial pellet was washed with 1 ml PBS twice and centrifuged again under the same conditions. After the second centrifugation the bacterial pellet was used for the DNA extraction. The DNA extraction was performed as recommended by the manufacturer (Wizard® Genomic DNA Purification Kit, Promega, USA). Purified bacterial genomic DNA obtained from each bacterial isolate was stored at -20°C.

2.5.2 Rapid DNA extraction

Bacterial colonies (2-3) were suspended in 100 µl of sterile dH_2O in a 1.5 ml microcentrifuge tube. The suspension was boiled in a block heater at 100°C for 5 min; following that it was incubated on ice for 5 min. The boiled bacterial suspension was centrifuged at 14000 x *g* for 3 min. The supernatant was used as DNA template for PCR reactions.

2.5.3 Determination of DNA concentration

Extracted bacterial genomic dsDNA was diluted in sterilised dH_2O. The DNA was diluted ~30 times and then was measured by a spectrophotometer or a NanoDrop 2000 at absorbance of 260 nm and 280 nm. The obtained concentration was adjusted to the required concentration for each experiment.

2.6 Oligonucleotides (primers)

2.6.1 Primers design

The Clone Manager 7 software (Scientific & Educational Software, USA) was used for designing the primers following its default standard criteria (GC: 50-60%, Tm: 55-80°C, 3' dimers: < 3 matches 3'end, dimers-any: < 7 adj homol bases, GC clamp: > 1 G or C at 3' end, stability: > 1.2 kcals 5'vs3', repeats: < 3 dinuc repeats, hairpins Annealing: 55°C and length: 18-30 bp). The NCBI data-base site was used to find *C. concisus* 13826 genome sequence and to test the specificity of the primer sets. Most primers sets were designed according to *C. concisus* 13826 genome sequence unless otherwise mentioned. This sequence was the only one available in public databases at the commencement of the study.

2.6.2 Primer sets used in this study

The primers designed and used in all chapters of this study are described in Table 2.2.

2.7 PCR amplification conditions

A total reaction volume of 25 µl contained 10 pmol of each primer set (GeneWorks), 20 ng bacterial DNA, 0.7 U of Taq polymerase. 'MyTaqTM' DNA Polymerase mixture (Bioline, Australia) was used as the PCR reaction solution.

Thermal cycling conditions for the targeted DNA, unless otherwise indicated, were: 2 min at 95°C, then thirty cycles of 30 seconds at 94°C, 30 seconds at 54°C and 2 min at 72°C, followed final extension of 7 min at 72°C with the exception of the extension temperature for primer set VI was 68°C and the annealing temperature for DnaJT and CjaAT primer sets was 58°C and extension time was 1 min.

Table 2.2: The sequence and targeted DNA regions of primer sets used in this study.

Primer sets	Primer sequence (5` to 3`)	Target region on the genomic DNA	Product size (bp)	Reference	Usage of primers in chapters
MUC1	F-ATGAGTAGCGATAATTGGG	23S rDNA		Bastyns *et al.*	3 & 5
CON1	R-CAGTATCGGCAATTCGCT	23S rDNA	306	(1995)	
CON2	R-GACAGTATCAAGGATTTACG	23S rDNA	308		
Pcisus5-F	F-AGCAGCATCTATATCACGTT	*Gyrase B*	344	Matsheka *et al.*	3 & 5
Pcisus6-R	R-CCCGTTTGATAGGCGATAG			(2001)	
Primer set (I)	F-TGGATACTCGGCCTTCATCTTG	CCC13826_0963	800	This study	3 & 5
	R-ATCTCTTGGCCTAGCAGCTC			This study	
Primer set	F-GCCAAGCGCCATGTCATAAG	CCC13826_0963	300	This study	3, 4 & 5
(II)	R-TGAATTTGGCGGCGGCAAAG			This study	
Primer set	F-GAGATCGTTGAGTCTCAAAG	*cjaC &* shikimate	330	Istivan	3
(III)	R-TATATCAAGCGGTCTTAGC	5-dehydrogenase		(Unpublished data)	
Primer set	F-CTATCTATAATAGCCACCAGTA	*cjaC* & shikimate	270	Istivan	3
(IV)	R-GTTGAGTCTCAAAGAGCTTTC	5-dehydrogenase		(Unpublished data)	
Primer set	F-TCCTTCTCCAGCTTCTCATC	CCC13826_0962	330	This study	3
(V)	R-AGGCCGAGTATCCAGACATC	& CCC13826_0963		This study	
Primer set	F-CTCTTGCAGTAGTAACCTTAG	CCC13826_0962	600	This study	3
(VI)	R-AAGCGGCTGCTGCTGAAGTG	& CCC13826_0963		This study	
CjaA	F-TAGAGTTTGCCGCCTTCGAC	CCC13826_0664	1200	This study	3
	R-ATGTGGCTAAGCAAGGTTGG	& CCC13826_0663			
CjaAT	F-GGCGTTATCATGCGCTAGAG	CCC13826_0664	299	This study	3 & 4
	R-GCTGACAAGGTCGATATCAC				
DnaJ	F- TCTCGTCGGTGCTTGACTTG	CCC13826_0965	900	This study	3
	R- AAACGCTGGTGACAAAGAGG				
DnaJT	F-CGTAAAGATCGCCTTGAACG	CCC13826_0965	304	This study	3 & 4
	R-GCCTTGCCCAACATGTAACG				
ZoT	F-TGCAAACCCTTTGTGATGAA	CCC13826_2276	355	Kalischuk *et al.*	3, 4 & 5
	R-CATGAGCCAGCTCAATCAAC			(2011)	
518R[a]	R-ATTACCGCGGCTGCTGG		170	Muyzer *et al.*	3
341F-GC	F-CCTACGGGAGGCAGCAG	CCC13826_2336		(1993) Muyzer	
907R	R-CCGTCAATTCMTTTGAGTTT		562	*et al.* (1998)	
C412-F	F-GGATGACACTTTTCGGAGC	16S rDNA	816	Linton *et al.*	5

C1288-R	R- CATTGTAGCACGTGTGTC	16S rDNA		(1996)	
HipO-F	F-GACTTCGTGCAGATATGGATGCTT	Hippuricase of	500	Persson *et al.*	5
HipO-R	R-GCTATAACTATCCGAAGAAGCCATCA	*C. jejuni*		(2005)	
CC18F	F-GGTATGATTTCTACAAAGCGAG	Aspartokinase of	416	Linton *et al.*	5
CC519R	R-ATAAAAGACTATCGTCGCGTG	*C. coli*		(1997)	
Concisus-F	F-CTTGTGAAATCCTATGGCTTA	16S rDNA	560	Man *et al.*	5
Concisus-R	R-CTCATTAGAGTGCTCAGCC	16S rDNA		(2010b)	
ZoT	F-TGCAAACCCTTTGTGATGAA	CCC13826_2276	1055	Mahendran *et*	5
ZotA2-R	R-TCGGTCCTCCACGATCTG			*al.* (2013)	
ZotB-F	F-TTGAGCTGGCTCATGCTTAG	CCC13826_2276	300	This study	5
ZotB-R	R-GTCTAAGCGTCAGACAAATAGAG	CCC13826_2276			

[a]: Muyzer primer set 1 (341F-GC and 518R and). Muyzer primer set 2 (341F-GC and 907R).

2.8 Preparation of DNA samples for sequencing

DNA purification from agarose gels and PCR reactions for sequencing DNA was by Isolate II PCR and Gel kit (Bioline, Australia) as recommended by the manufacturer. The sequencing was performed by Australian Genome Research Facility (AGRF). The DNA amount was adjusted according to AGRF instructions based on the DNA length. The DNA was made up to a volume of 11 µl in Nuclease-Free Water (Qiagen, Australia) and to this was added 1 µl of either forward or reverse primer. The mixture of the DNA and primer (12 µl) was sent to AGRF by post (it took approximately 16-22 h to be received by AGRF).

2.9 Gel electrophoresis techniques

2.9.1 Loading buffer (11x)

The loading buffer was made by dissolving Tris-HCl (50 mM) in sterilised dH2O at pH 8.0. Then this solution was added EDTA (10 mM), ficol-400 (10% v/v), SDS (1% w/v), Orange G dye (0.5% w/v) and glycerol (50% v/v). A volume of 1.0 ml was aliquoted into 1.5 ml tubes and stored at room temperature.

2.9.2 λ DNA (*Pst*I) marker

The λ DNA marker was a mixture of 900 µl sterile dH_2O, 90 µl buffer H (10X), 108 µl λ DNA (50 µg) and 18 µl *Pst*I (100 U/µl). The mixture was then incubated overnight at 37°C. The mixture was added to 90 µl of loading dye prior to use. The whole mixture was aliquoted into 200 µl volumes in 1.5 mm tubes and stored in -20°C.

2.9.3 TAE Buffer (50x)

The TAE buffer was prepared by dissolving a mixture of glacial acetic acid (5.17% w/v), EDTA (1.86% w/v) and Tris-base (24.2% w/v) in dH_2O and then diluted to 1x before use.

2.9.4 Preparation of agarose gel and DNA electrophoresis

Agarose powder (1.5% w/v) was dissolved into 1x TAE buffer and boiled until completely dissolved. Two gel sizes were used: mini-gels (50 ml) of 1x TAE and midi-gels (100 ml). The boiled agarose was allowed to cool on a shaker to reach approximately 55°C. It was then decanted into a casting tray and allowed to solidify for 30 min when it was ready for use.

A volume of 11x loading dye was added to 10 volumes of each DNA sample and mixed well. The mixture of DNA sample with loading dye (12 µl) was loaded into the prepared gel well. Electrophoresis was then performed at voltages of 100-120 V using a power supply unit for the required time period. The DNA gel was stained in ethidium bromide solution at a concentration of 1 mg/ml for 15 min. The DNA gel was destained by washing in a stream of tap water. The gel was visualised by a UV transilluminator to detect DNA bands, and photographed by the Gel-Doc System.

2.10 Denaturing gradient gel electrophoresis (DGGE) technique

2.10.1 Acrylamide denaturant (100%)

Every time a gel was run, a fresh mixture was prepared consisting of 40 ml acrylamide/bis-acrylamide (40%) 37.5:1, 84 g urea, 80 ml formamide (100%), 2 ml TAE (50x) made up to 200 ml in dH_2O. The catalysts, TEMED (100 µl) and ammonium persulfate (APS) (2 ml of a 10% solution) were then added to the mixture to polymerise acrylamide cross-linkage and form a solid gel.

2.10.2 TAE stock (50x)

TAE stock was prepared by mixing 242 g tris-base, 57.1 ml glacial acetic acid, 100 ml EDTA (0.5 M) and made up to 1 L with dH_2O.

2.10.3 DGGE solutions

1- Acrylamide/bis-acrylamide (6%)

Chemicals	**Denaturant (0%)**	**Denaturant (70%)**
Acrylamide/bis-acrylamide 40%, 37.5:1	15 ml	15 ml
Formamide	-	28 ml
Urea	-	29.4 g
TAE (50x)	2 ml	2 ml
dH_2O	Up to 100 ml	Up to 100 ml

2- Acrylamide/ **bis-acrylamide (12%)**

Chemicals	**Denaturant (0%)**	**Denaturant (70%)**
Acrylamide/bis-acrylamide 40%, 37.5:1	30 ml	30 ml
Formamide	-	28 ml
Urea	-	29.4 g
TAE (50x)	2 ml	2 mL
dH_2O	up to 100 ml	Up to 100 ml

2.10.4 Preparation of staining solution

Solutions	Chemicals quantity
Fixing Solution I	50 ml ethanol (100%) was mixed with 2.5 ml glacial acetic acid and 447.5 ml dH_2O.
Silver nitrate (prepared fresh)	0.2 g silver nitrate was dissolved in 200 ml dH_2O.
Developing solution (prepared fresh)	0.02 g sodium borohydride and 3 g sodium hydroxide were dissolved in 200 ml dH_2O containing 0.8 ml formaldehyde.
Fixing Solution II	3.75 g sodium carbonate was dissolved in 500 ml dH_2O.
Preservative solution	A mixture of 125 ml ethanol (100%), 50 ml glycerol and 325 ml dH_2O.

2.10.5 DGGE PCR conditions and solutions

The DGGE PCR was performed in a 50 µl PCR reaction and PCR thermal cycling environments were as previously described (Muyzer *et al.* 1993; Muyzer *et al.* 1998). For Muyzer primer set 1 (341F-GC & 518R), the reaction started with five min at 94 ºC then four cycles of 30 seconds at 95°C, 30 seconds at 55°C and 1 min at 72°C, followed by 25 cycles of 30 seconds at 92°C, 30 seconds at 55°C and 1 min at 72°C. Finally the reaction was terminated with 10 min at 72°C. For Muyzer primer set 2 (341F-GC & 907R), the PCR conditions were 94°C for 5 min, followed by 20 cycles of 94°C for 1 min, 65°C to 55°C for 1 min (touchdown -0.5°C/cycle), and 72°C for 3 min, followed by 15 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min, followed by final extension of 7 min at 72°C. The PCR results were analysed by DGGE.

2.10.6 DGGE

DGGE analysis was performed by loading 15 µl of PCR product into 6% and 12% (w/v) polyacrylamide gels that were prepared as a denaturing gradient from 45%-65% (each 100% denaturant was comprised of 40% formamide and 7 M urea). Electrophoresis was performed in 1x TAE buffer at 60 V for 18 h at 60°C. The gel was then stained with silver nitrate and fixed for 2 h in 200 ml solution I. After that, the gel was incubated in a 200 ml the silver nitrate solution for 20 min. The DGGE gel was then developed in 200 ml of a solution containing 3 g sodium hydroxide, 0.8 ml formaldehyde and 0.02 g sodium borohydride for 30 min and then incubated in another fixing solution II for 10 min. The staining was finalised by soaking the gel in 200 ml the preservative solution for 10 min. The DGGE gel was visualised with EPSON Expression scanner 1600 V.2.65 E software 2.9.

2.11 Tissue culture techniques

All tissue culture work was performed in a class II biological safety cabinet to reduce contamination risks. Media and solutions were stored in reusable containers. Glassware was cleaned using Pyroneg detergent, washed at least twice with dH_2O and finally immersed in dH_2O overnight. The containers were then autoclaved and used to store tissue culture solutions. The sterile containers were re-autoclaved with unsterilised solutions before use in tissue culture.

2.11.1 Dulbecco's Modified Eagle Medium (DMEM)

The modified Dulbecco's Eagle Medium (DMEM) was made by adding 10 ml of HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer to a bottle containing 500 ml DMEM. The DMEM with HEPES was aliquoted into 50 ml conical tubes (45 ml of DMEM/tube) and stored at 4°C. Before use, the medium was supplemented with filtered

heat-inactivated FBS (10% v/v) in the conical tube (DMEM/FBS). This medium was used for the growth of the INT407 cell line.

2.11.2 Triton X-100 (detergent) 0.3%

A working solution of triton X-100 (0.3% v/v) was made by dilution in sterile PBS. The solution was sterilised by filtration using a small size filter (0.22 µm) and stored at 4°C.

2.11.3 Tissue culture growth environments

A frozen stock of INT407 cells in liquid nitrogen was resuscitated by thawing and inoculating into a flask containing DMEM/FBS. INT407 cells were incubated in a CO_2 incubator at 37°C. INT407 cells were allowed to grow to become confluent (80-95%). The growth was then split into a new flask for use in the experiment or collected for a new stock. INT407 cells were passaged no more than 10 times before they were discarded prior to a new stock being cultured.

2.11.4 Seeding and maintaining the intestinal cell line (INT407)

For seeding INT407 cells in new flasks, the old medium was discarded and the cells were washed with PBS twice. Then the cells were dissociated by adding trypsin-EDTA (1 ml for 25 ^{2}C flasks or 3 ml for 75 ^{2}C flasks) and incubated at 37°C for 10 min. The dissociated cells were transferred to a centrifuge tube. The cells were washed twice in DMEM without FBS to remove the trypsin-EDTA solution. After each wash, the cells were resuspended in 5 ml DMEM and centrifuged at 1000 x *g* for 3 min. The supernatant was discarded at each of washing. After the last washing, the pellet of cells was resuspended in the appropriate amount of DMEM/FBS. The cells were counted and used for either seeding or making stocks.

The stocks of INT407 cells were made in cryovials from the cleaned resuspended cells in DMEM/FBS with 10% dimethyl sulfoxide (DMSO), as a cryoprotectant. The cryovials were first stored at -80°C for 48 h and then transported and stored in liquid nitrogen.

Cell counts were determined by Countess® Automated Cell Counter (Invitrogen, Australia) following the manufacturer's instructions. The following is a brief outline of the procedure using resuspended cells. The first step was to mix 10 µl of resuspended cells with an equal amount of 0.4% trypan blue stain. Then, from the mixture, 10 µl was loaded into a counting chamber. The count obtained for the total number of viable cells was used to obtain the desired experimental cell concentration. Dilutions were prepared based on calculations made by the cell counter.

2.11.5 Invasion and adhesion assays

The capability of *C. concisus* isolates to adhere to and invade INT407 cells *in vitro* was investigated according to the method previously described by Elsinghorst (1994) and then modified by Chang (2002), RMIT University with some modifications. In brief, the preparation of INT407 cells was as follows: INT407 cells were seeded in large flasks (75 cm^2) and allowed to reach a semi-confluent monolayer in 2-3 days. The cells were harvested from the flask, counted and the cell viability was determined by staining with trypan blue. The viability of cells used in these assays was >95%.

The INT407 cells were seeded in a 24 well microtiter plate at 10^5 cells per well. The plates were incubated for 12-16 h to allow the cells to attach. On the day of the assays, the old medium was discarded and the cells were washed three times with PBS. After washing, 400 ml of fresh DMEM/FBS were added to each well before adding the bacterial cells.

Bacterial cells were prepared by selecting 5-7 colonies from 48 h old bacterial growth on HBA and suspending in Columbia broth. The OD_{600} value for the bacterial suspension was

adjusted to 0.1, which is equivalent to 10^8 CFU. Then 100 µl from this bacterial suspension were inoculated into each well containing 10^5 INT407 cells to obtain multiplicity of infection (MOI) at ~100. The plates were centrifuged at 160 x *g* for 5 min to enhance the bacterial contacts with INT407 cells. The inoculated plates were incubated for 6 h at 37°C under *C. concisus* growth conditions (section 2.4.2) to allow the bacteria to adhere to and invade INT407 cells.

The adhesion assay was performed by washing the plates three times with PBS after 6 h incubation to remove the non-adhering/non-invading bacterial cells. To release the adherent and intracellular bacterial cells, INT407 cells were lysed by adding 200 µl of 0.3% triton-X-100 in each well. Plates were incubated in 5% CO_2 at 37°C for 15 min. The cell lysate was diluted by adding 800 µl PBS. To retrieve the viable bacterial cell number, the CFU was determined and the percentage of adhered cells was calculated by the following equation:

$$\text{Adhesion } \% = \frac{\text{no. of bacterial cells retrieved/ml}}{\text{no. of bacterial cells initially used/ml}} \times 100$$

Invasion was determined by the gentamicin protection assay as follows: plates were washed three times with PBS to remove the non-adhering/non-invading bacterial cells. Then 1 ml of DMEM/FBS containing 400 µg/ml gentamicin was added to each well and the plates were incubated in 5% CO_2 at 37°C for 90 min to kill bacterial cells that had not invaded; since gentamicin is bactericidal. After gentamicin treatment, the plates were washed three times with PBS to remove gentamicin. The INT407 cells were lysed by 0.3% triton-X-100 to release the invaded bacterial cells. To ensure the bacterial cells were susceptible to the gentamicin concentration used, DMEM/FBS containing 400 µg/ml gentamicin was added to wells containing bacterial cells in DMEM/FBS without INT407 cells. Finally, to retrieve the bacterial viable cell number, the CFU was determined and the percentage of invaded cells was calculated by the following equation:

$$\text{Invasion } \% = \frac{\text{no. of bacterial cells retrieved from gentamicin} - \text{treated cells/ml}}{\text{no. of bacterial cells initially used/ml}} \times 100$$

To evaluate the invasiveness of *C. concisus* isolates, the invasive index was calculated by dividing the number of internalised *C. concisus* cells by the number of adherent *C. concisus* cells multiplied by 100 (Larson *et al.* 2008).

$$\text{invasive index value} = \frac{\text{No. of invaded bacteria (\%)}}{\text{No. of adherent bacteria (\%)}} \times 100$$

2.12 Gene expression studies

2.12.1 RNA techniques

All RNA experiments were performed in a class II biological safety cabinet to avoid any environmental contaminants such as RNases. Reusable items like glass bottles were treated with diethylpyrocarbonate (DEPC) before autoclaving to eliminate any chance of contamination with nucleases. All solutions used for RNA methods, were prepared in RNase-free water. The prepared solutions were stored in DEPC-washed containers or in plastic sterilised RNase-free containers. All disposable plastic ware used during RNA techniques was sterile and RNase-free. Pipettes were sterilised before use and tips were sterilised RNase-free filter. Glassware and plastic ware was sterilised prior to each experiment using UV for 20 min.

2.12.2 TE buffer

TE buffer was prepared by dissolving EDTA (1 mM) and Tris-base (10 mM) in RNase-free water. The pH was then adjusted to 8 and the buffer was sterilised using a 0.22 µm filter.

2.12.3 RNA extraction

The RNA extraction was performed by the 'Isolate RNA Mini' kit (Bioline, Australia) following the instructions provided by the manufacturer.

Extracted RNA of each isolate was treated with DNase to remove any residual genomic DNA using the 'Turbo DNA-free^{TM}' kit (Ambion) following the instructions of the manufacturer with the exception of the incubation at 37°C was for 60 min instead of 30 min to degrade residual genomic DNA.

2.12.4 cDNA synthesis

cDNA synthesis was performed with the same amount of RNA (1 µg) for each strain. The cDNA was synthesised using the 'Tetro cDNA Synthesis' kit (Bioline, Australia) as recommended by the manufacturer with the exception of the incubation at 45°C for 55 min instead of 30 °C min.

2.12.5 RT-PCR conditions

Semi-quantitative SYBR green real time PCR was performed using the 'SensiFAST^{TM} SYBR No-ROX' kit (Bioline, Australia) in the Qiagen Rotagene (Qiagen). A total mixture of 12 µl reaction contained of 6 µl of 2X SensiFAST SYBR No-ROX Mix, 5 pmol of each primer set (GeneWorks) and 5 µl template (1 ng of cDNA) with the exception of primer set I, 2.5 pmol was used instead of 5.

Thermal cycling conditions were in accordance with the manufacturer's instructions with some modifications as follows: 5 min at 95ºC for hot start Taq DNA polymerase activation, forty cycles of 15 sec at 95°C, 15 sec at 55°C and 60 sec at 72°C.

The melting curves were generated by ramping the temperature from 45-95°C in two steps as follows:

- Step1 (pre-melting), the temperature ramp rate was one degree every 90 sec.
- Step 2 (melting point) the temperature ramp rate was one degree every 5 sec.

Primer efficiency was determined, and relative gene expression, normalised to the housekeeping gene, was determined using the method described by Pfaffl (2001).

2.13 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.13.1 Lowry assay reagents

The preparation of Lowry assay reagents was as described by Markwell *et al.* (1978) summarised by the following steps:

I. **Reagent A:** sodium carbonate (2% w/v), sodium hydroxide (0.4% w/v), sodium potassium tartrate (0.16% w/v) and SDS (1% w/v) were dissolved in dH_2O.
II. **Reagent B:** Copper sulphate (4% w/v) was dissolved in dH_2O.
III. **Reagent C:** A mixture of reagent A (50 ml) and reagent B 0.5 ml. This solution was freshly prepared before use.
IV. **Reagent D:** Folin-ciocalteu reagent was diluted (1:1) in dH_2O.

2.13.2 SDS sample buffer (5x) solution (for loading the protein into gels)

The SDS sample buffer (5x) solution was a mixture of glycerol (25% v/v), bromophenol blue (0.1% w/v), SDS (2% w/v) and β-mercaptoethanol (14.4 mM v/v) in a solution of Tris-HCl (60 mM) at pH 6.8. The storage of this solution was at -20°C.

2.13.3 SDS-PAGE running buffer (10x)

The SDS-PAGE running buffer (10x) was prepared by dissolving tris-base (3% w/v), glycine (14.4% w/v) and sodium dodecyl sulfate (SDS) (1% w/v) in dH_2O. The pH of the mixture was adjusted to 8.3 and it was diluted to 1x before use.

2.13.4 Coomassie brilliant blue stain preparation

The Coomassie brilliant blue stain was made by mixing Coomassie brilliant blue (0.05% w/v) in methanol (40% v/v), glacial acetic acid (10% v/v) and dH_2O (50% v/v).

2.13.5 Cell lysate buffer

The cell lysate buffer was prepared by dissolving 2 g of SDS (2% w/v), 15 ml of glycerol (15% v/v) and 35 mg of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Pefabloc® SC) (2mM) in 85 ml of Tris-HCL (0.1M) at pH 6.8.

2.13.6 De-staining solution

The de-staining solution was made by papering a mixture of glacial acetic acid (10% v/v) and methanol (40% v/v) in dH_2O.

2.13.7 Whole cell lysate preparation

C. concisus isolates were grown on HBA plates for 48 h. The entire bacterial growth on a HBA plate was harvested and suspended in 1 ml of 10 mM Tris-HCL. The suspension was centrifuged at 14,000 x *g* for 2 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 10 mM Tris-HCL. The suspension was then precipitated by centrifugation at 14,000 x *g* for 2 min. To lyse the bacterial cells, the supernatant was discarded and 500 µl of cell lysate buffer was added to the pellet. The mixture (cell pellet and

lysate buffer) was incubated at 100°C for 3-5 min (without boiling the lysate). The mixture was then centrifuged at 14,000 x *g* for 5 min and the supernatant (containing the whole cell lysate) was transferred to a clean tube and stored at -20°C.

2.13.8 Protein estimation for whole cell lysate

The protein concentration of whole cell lysates was determined by the modified Lowry method as described by Markwell *et al.* (1978) using a spectrophotometer. A standard curve was constructed by performing several dilutions from standard albumin as follows: 0, 10, 20, 30, 40, 50, 70 and 100 µl of protein standard (1 mg/ml) and completed with sterile dH_2O to make a final volume of 200 µl. For bacterial lysate, 10 µl was added to 190 µl of dH_2O to make the same final volume of 200 µl. The blank was 10 µl of cell lysate buffer (without bacterial cells) added to 190 µl of dH_2O.

Reagent C (600 µl) was added to all tubes and the mixtures were incubated at room temperature for 20 min. Reagent D (60 µl) was then added, mixed and the mixture incubated at room temperature for 30 min. From each tube, 200 µl was aliquoted (in triplicate) into a 96 plate and the absorbance was measured by a spectrophotometer at 600 nm (OD_{600}). From the absorbance values, the standard curve was plotted using Microsoft Excel and the protein concentration of the samples was calculated by the equation obtained from the standard curve.

2.13.9 Preparation of the protein sample for gel electrophoresis

Protein samples were prepared as described by Laemmli (1970). Bacterial whole cell lysate protein was adjusted to a concentration of 10 µg/µl by diluting the lysate in sterile dH_2O. Then, 10 µl was mixed with 5 µl of 5x-SDS sample buffer solution. The mixture was denatured at 95°C for 5 min and allowed to cool to room temperature. The tubes were

centrifuged and then the mixture was loaded into individual wells of SDS-PAGE gel by a 1-100 µl micro-syringe. Protein standard (Precision Plus, Bio-Rad) was used as a marker.

2.13.10 SDS-PAGE gel composition

An SDS-PAGE gel with an acrylamide concentration of 12.5 % w/v was used as a resolving gel. An SDS-PAGE gel with an acrylamide concentration of 4.5% w/v was used as the stacking gel.

The preparation of the resolving gel (12.5%) was as following:

Chemicals	**Concentration**	**volume/ gel**
dH2O		4.22 ml
Tris-HCl (pH 6.8)	1.5M (w/v)	2.5 ml
SDS	10% (w/v)	0.1 ml
Acrylamide (38.67%)/ Bis-Acrylamide(1.33%)	40% (w/v)	3.135 ml
APS (fresh) ammonium persulfate	10% (w/v)	0.05 ml
TEMED (N,N,N,N-teramethylenediamine)		0.01 ml

The preparation of the stacking gel (4.5%) as following:

Chemicals	**Concentration**	**volume/ gel**
dH2O		3.11 ml
Tris-HCl (pH 6.8)	0.5 M (w/v)	1.25 ml
SDS	10% (w/v)	0.05 ml
Acrylamide (38.67%) + Bis-Acrylamide (1.33%)	40% (w/v)	0.563 ml
APS (fresh)	10% (w/v)	0.025 ml
TEMED		0.005 ml

SDS-PAGE electrophoresis was performed using a Mini-PROTEAN Tetra Cell electrophoresis system. Protein gel electrophoresis was achieved with two steps: step I was at 60 V for 30 min and step II was at 180 V for 60 min.

2.13.11 SDS-PAGE gel staining and de-staining

The gel was transferred and immersed into a container with Coomassie brilliant blue solution on a shaker for 1 h. The gel was then immersed in de-staining solution overnight. The SDS-PAGE gel was then photographed using a scanner. The gel was stored in 5% acetic acid at 4^{o}C.

Chapter 3 : Genetic diversity of *C. concisus* and detection of putative virulence genes

3.1 Introduction

Although *C. concisus* has been detected in several infected sites in humans, it has not been recognised as a pathogen. It is not clear whether all *C. concisus* strains or specific groups are associated with human infections. When the work described in this chapter commenced, there was only one sequenced *C. concisus* strain available (13826) (Fouts *et al.* 2007). The genome of *C. concisus* 13826 contains several genes similar to virulence genes found in the *C. jejuni* genome (Kalischuk *et al.* 2011; Pawelec *et al.* 2000; van Vliet *et al.* 2001). The sequence of this strain was used to design most of the primers sets to amplify putative virulence genes. Later, several other sequenced *C. concisus* strains became available in the NCBI database (Deshpande *et al.* 2011; Deshpande *et al.* 2013) and this sequence information was used in a bioinformatics study in order to understand the possible genetic variations between *C. concisus* isolates.

The Muyzer primer sets that amplify 16S rDNA were originally designed to detect and analyse the genetic diversity of mixed bacterial populations in environmental samples such as soil and water using the PCR-DGGE technique (Muyzer *et al.* 1993; Muyzer *et al.* 1998). This technique was also used to detect and group extremely fastidious bacteria including *C. concisus* using either pure or mixed DNA (section 1.9.3) (Cornelius *et al.* 2012; Petersen *et al.* 2007).

An earlier study in our laboratory showed that the sequence of a DNA fragment from a *C. concisus* isolate obtained from a patient with diarrhoea (RCH3) partially contained the sequences of *cjaC* and followed by shikimate 5-dehydrogenase gene according to alignment

with strain 13826, but the two genes are separated in the genome of strain 13826. Primers for amplification this fragment were designed based on the sequence of *C. concisus* RCH3 (Istivan, *unpublished* data). The presence of *cjaC* and other putative virulence genes such as *cjaA*, *dnaJ* and *zot* has not been fully investigated in *C. concisus* isolates (section 1.10.2).

The aims of the experiments conducted for this chapter were to:

(i) Confirm the identity of *C. concisus* oral and faecal isolates used in this study.

(ii) Apply PCR-DGGE to a collection of oral and faecal *C. concisus* isolates using the two commonly used Muyzer primer sets, in order to detect and type the isolates.

(iii) Investigate sequence differences in *cjaC* and its adjacent genes using primers based on DNA fragments obtained from *C. concisus* RCH3 and 13826 by PCR.

(iv) Search for the putative virulence genes (*cjaA*, *dnaJ* and *zot*) in the genomes of *C. concisus* isolates from the same collection of oral and faecal isolates by PCR using primers based on strain 13826.

(v) Perform bioinformatics analysis of putative virulence genes (*cjaC*, *cjaA*, *dnaJ* and *zot*) using the recently published *C. concisus* sequences to identify the presence and sequence difference of these genes.

3.2 Materials and Methods

3.2.1 Bacterial isolates and reference strains

The bacterial isolates and reference strains used in this chapter are described in section 2.4.1. PCR-DGGE typing was performed on 14 selected *C. concisus* isolates (ATCC 51561, ATCC 51562, RCH6, RCH3, RCH11, RCH15, RCH20, RCH26, O8, O9, O10, O11, O13 and O17) and the following bacterial reference strains were used as controls: *C. mucosalis* ATCC 43264, *C. jejuni* 81116, *C. coli* NCTC 11366 and *E. coli* ATCC 25922. All *C. concisus* isolates listed in Table 2.1 (section 2.4.1) were screened for the presence of *cjaC*, *cjaA*, *dnaJ* and *zot*.

3.2.2 Confirmation the identity of *C. concisus* isolates used in the study

Molecular identification was performed by two PCR amplification methods. The first PCR employed the Pcisus (5&6) primer set targeting the *GyrB* gene as was described by Matsheka *et al.* (2001), while the second PCR was performed by using primer set (MUC1 and CON1/CON2) targeting the 23S rDNA gene that was described by Bastyns *et al.* (1995). These primer sets amplifying the 23S rDNA gene were used to confirm the bacterial identity and assign *C. concisus* isolates into the genomospecies A and B as described by Istivan *et al.* (2004). The DNA extraction and PCR amplification protocols are described in sections 2.5 and 2.7, and the primer set sequences are listed in Table 2.2.

3.2.3 Typing *C. concisus* isolates by DGGE

DNA was extracted from the tested *C. concisus* isolates and *Campylobacter* reference strains growing on HBA. Then, two Muyzer primer sets were used for the PCR-DGGE (Muyzer 1, 341F-GC & 518R and Muyzer 2, 341F-GC & 907R) for amplifying segments of the 16S

rDNA gene for typing *C. concisus* isolates (Table 2.2 and Figure 3.1). Lambda DNA (*Pst*I) was used was used as a reference maker (section 2.9.2). The PCR products were analysed by DGGE as described by Muyzer *et al.* (1998). The obtained PCR products from all *C. concisus* isolates were also mixed together and analysed by DGGE. The PCR amplification and DGGE protocols are described in section 2.10. The results obtained from DGGE gels were analysed by Phoretix ID software (TotalLab, Newcastle upon Tyne, UK) to generate similarity dendrograms between the tested isolates and to group the lanes upon banding patterns using the Unweighted Pair Group method with Mathematical Averages (UPGMA) clustering. Sorenson's coefficient was used for pairwise calculation between the DNA bands.

3.2.4 Detection of *cjaC* and investigation of the RCH3 fragment sequence location

Six primer sets were designed to detect *cjaC* and to investigate the RCH3 fragment location in the genome of *C. concisus* 13826, as follows: primer sets I and II were designed based on strain 13826 DNA sequence and used to amplify parts of the *cjaC* gene (CCC13826_0963) (800 and 300 bp), primer sets III and IV were designed based on RCH3 sequence and used to amplify the RCH3 fragment (330 and 270 bp) from all *C. concisus* isolates, and primer sets V and VI were designed to investigate the location of *cjaC* (CCC13826_0963) and it is adjacent gene (CCC13826_0963) in *C. concisus* 13826. All these primer sets (six) were used to amplify the DNA regions from all *C. concisus* isolates listed in Table 2.1. The sequences of primer sets are described in Table 2.2 and their targeted regions are shown in Figure 3.2. The PCR conditions and protocols are described in section 2.7.

To investigate location of *cjaC* and its adjacent genes in several *C. concisus* isolates to be compared with *C. concisus* 13826, the obtained PCR products from amplifying the genomic DNA of selected *C. concisus* isolates (ATCC 51562, RCH3, RCH6, RCH26 and O11) were sequenced using primer sets I, II, III and VI.

3.2.5 Detection and identification of *cjaA, dnaJ* and *zot*

Based on the sequence of *C. concisus* 13826, CjaA primer set was designed to amplify part (1200 bp) of *cjaA* (CCC13826_0664) and its adjacent gene (glutamine transport ATP-binding protein GlnQ, CCC13826_0663) to investigate the location of *cjaA*. CjaAT primer set was designed to detect the *cjaA* gene (CCC13826_0664) (299 bp). DnaJ and DnaJT primer sets were designed to amplify 900 and 304 bp of *dnaJ* (CCC13826_0965), and the ZoT primer set was used to amplify 335 bp of *zot* (CCC13826_2276). The sequence of primer sets and their corresponding genomic regions are listed in Table 2.2 and shown in Figure 3.1. The PCR conditions and protocols are described in section 2.7.

3.2.6 Bioinformatics analysis following the recent publications on *C. concisus* sequences for *cjaC, cjaA* and *dnaJ*

This analysis was performed to provide an explanation for the failure of PCR amplification with some primer sets used in this study to amplify the genes in all of the *C. concisus* isolates and to examine the level of genetic variation within genomospecies A and B of *C. concisus* isolates. Gene sequence analysis was performed on *cjaC*, *cjaA* and *dnaJ* of the fully sequenced *C. concisus* strains available in the NCBI data-base site as described in section 1.9.4 and Table 1.3. For *cjaC*, DNA and amino acid sequences obtained from RCH6 were also aligned with sequence of all published *C. concisus* strains.

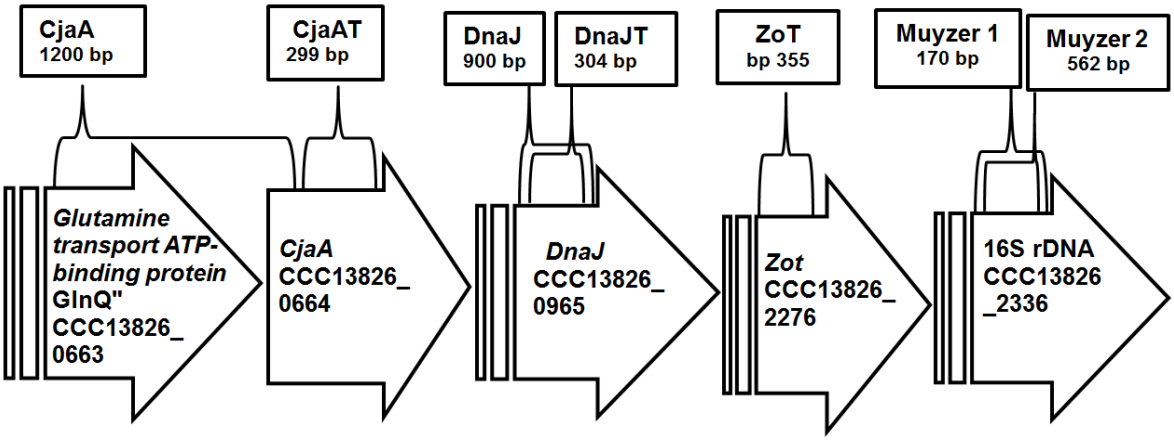


Figure 3.1: Genomic mapping of the designed primers sets (CjaA, CjaAT, DnaJ, DnaJT, ZoT, Muyzer 1 and Muyzer 2). The arrows represent the targeted genes based on the genome of *C. concisus* strain 13826. Intermittent boxes in start of arrows refer to that the genes (*cjaA, dnaJ, zot* and 16S rDNA) are apart in the genome of strain 13826.

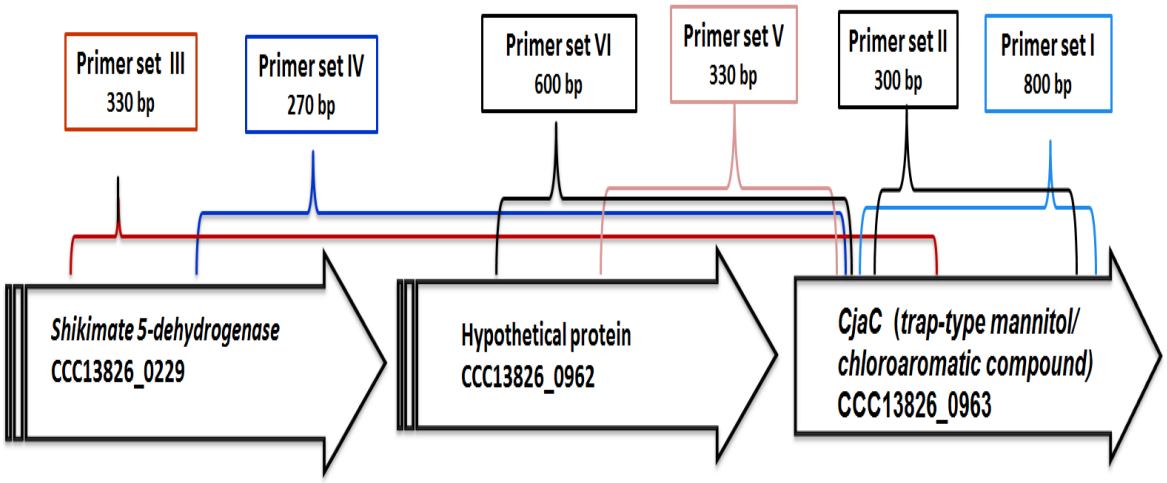


Figure 3.2: Genomic mapping of the primer sets designed and used to amplify the *cjaC* gene and its flanking region based on *C. concisus* reference strain 13826 genome. The arrows represent the targeted genes on the genome of *C. concisus* strain 13826. Intermittent boxes in the start of arrows refer to that the genes (shikimate 5-dehydrogenase and the hypothetical protein genes) are apart in the genome of strain 13826. The targeted DNA regions for primer sets III and IV were designed based on RCH3 DNA sequence.

3.2.7 Softwares used for data analysis

The NCBI data-base site was used to obtain and align the DNA and amino acid sequences of the genes of interest in sections 3.2.4, 3.2.5 and 3.2.6. ExPASY was used to translate nucleotide sequences to amino acid sequences (section 3.2.6) (Artimo *et al.* 2012). Molecular evolutionary genetics analysis (MEGA) software was used for the gene sequence alignments and generation of phylogenetic trees by the neighbour-joining dendrogram (Felsenstein 1989). For alignment and phylogenetic analysis of DNA and amino acids, default parameters were used as follows: for DNA, gap opening penalty of 15 and gap extension penalty of 6.66 were used. For amino acids, opening penalty of 10, gap extension penalty of 0.2, gap separation distance of 4 and protein weight matrix of Gonnet were used.

3.3 Results

3.3.1. Confirmation of the identity of *C. concisus* isolates

Although a PCR product was obtained from all *C. concisus* isolates DNA with either MUC1/CON1 (genomospecies A) or MUC1/CON2 (genomospecies B), the Pcisus (5 & 6) primer set failed to amplify a PCR product from the DNA of one *C. concisus* isolate (RCH20).

3.3.2 Typing *C. concisus* by DGGE

Using Muyzer primer set 1 (518R & 341F-GC), a PCR product was obtained from the DNA of all isolates and reference strains (Appendix I). The Phoretix ID software assigned all *Campylobacter* spp. isolates to four distinct groups that were defined as group I, II, III and IV (Figure 3.3 and Table 3.1). All *C. concisus* genomospecies B isolates fell into group II. Most of genomospecies A isolates were allocated to group I by DGGE, while three (RCH3, RCH26 and O17) were allocated to group II by DGGE. The mixture (Mix) of the DNA products also

presented the isolates in two separate bands (two groups) (Figure 3.3). *C. mucosalis* (ATCC 43264) represented a distinct group (III), while *C. jejuni* (81116) and *C. coli* (NCTC 11366) were both allocated to group VI. Moreover, *E. coli* (ATCC 25922) showed a unique band, which partly covered by smudge (see the black arrow) as can be seen in Figure 3.3 and Table 3.1.

Using of the second primer set (Muyzer primer set 2, 341F-GC & 907R), a PCR product was also obtained from all isolates (Appendix II). The Phoretix ID software used to analyse the DGGE gels defined four groups that were also designated I, II, III and IV (Figure 3.4); however distribution of isolates and reference strains between groups differed from the distribution generated by Muyzer primer set 1 (Table 3.1). The *C. concisus* isolates fell into two groups (I & II), with all those *C. concisus* isolates belonging to genomospecies B in group II and most genomospecies A isolates in group I. *C. concisus* strains ATCC 51562 and RCH20 (both from genomospecies A) fell into group II. The mixture (Mix) of the DNA products also presented the isolates in two separate bands (two groups) (Figure 3.4). *C mucosalis* ATCC 43264 fell into group I and *C. jejuni* 81116 and *C. coli* (NCTC 11366 into group III & IV respectively (Table 3.1 and Figure 3.4).

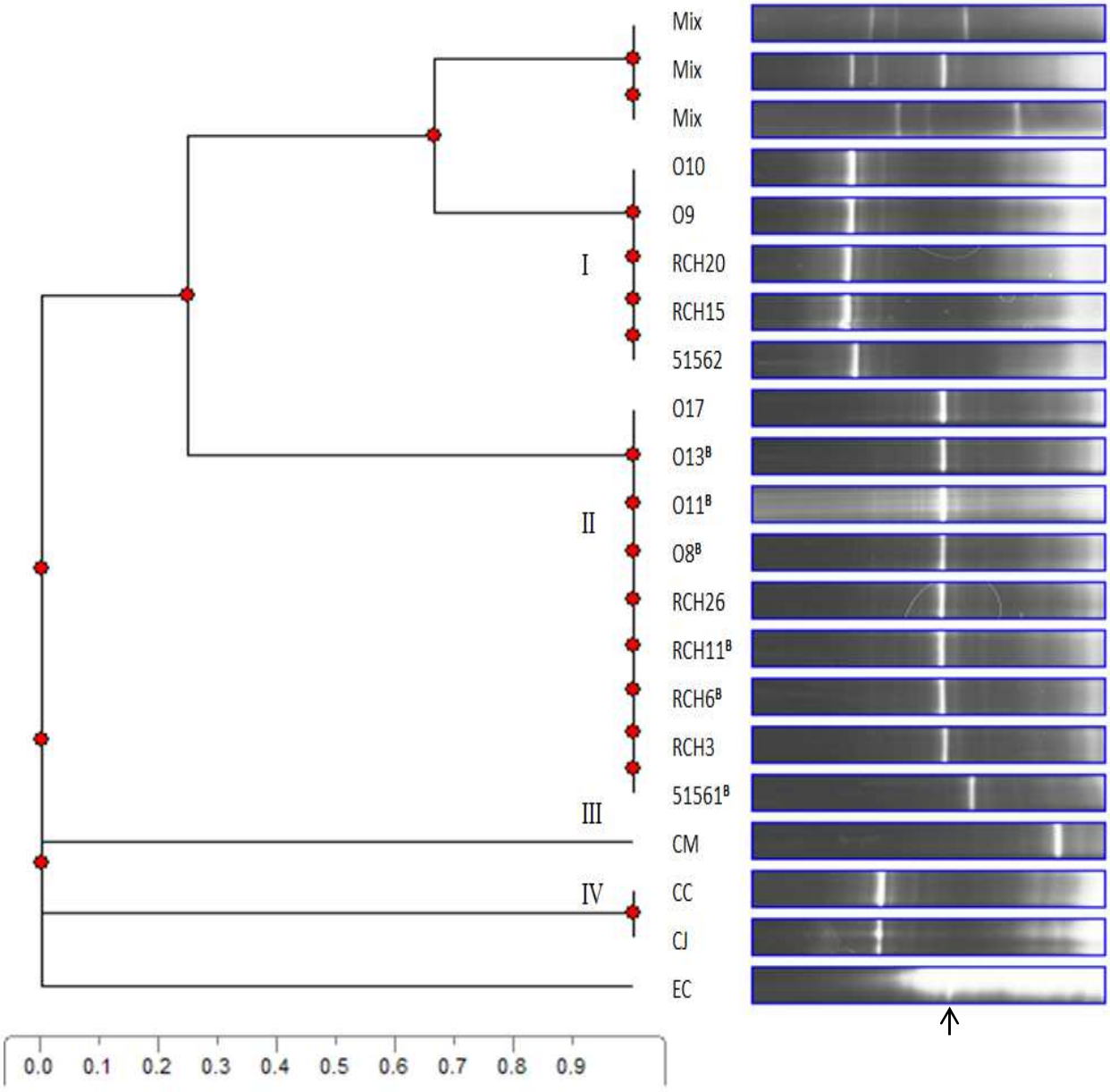


Figure 3.3: PCR-DGGE dendrogram using Muyzer primer set 1 (341F-GCA & 518R) targeting the 16S rDNA gene in *C. concisus* and other bacterial reference strains. Dendrogram analysis was generated by Phoretix ID software to analyse DGGE gel results. Mix: DNA mixtures from all *C. concisus* isolates used. 51561 and 51562 are *C. concisus* ATCC strains. RCH: clinical *C. concisus* isolates obtained from children with diarrhoea at the Royal Children's Hospital. O: *C. concisus* oral isolates from healthy volunteers. CM: *C. mucosalis* (ATCC 43264). CJ: *C. jejuni* (81116). CC: *C. coli* (NCTC 11366). EC: *E. coli* (ATCC 25922). I-V are bacterial groups generated from DGGE gel analysis by the software. ^{B}: *C. concisus* isolates belonging to genomospecies B. Other *C. concisus* isolates not marked as ^{B}, belong to genomospecies A. The black arrow is to show the location of *E. coli* band as it partly covered by smudge.

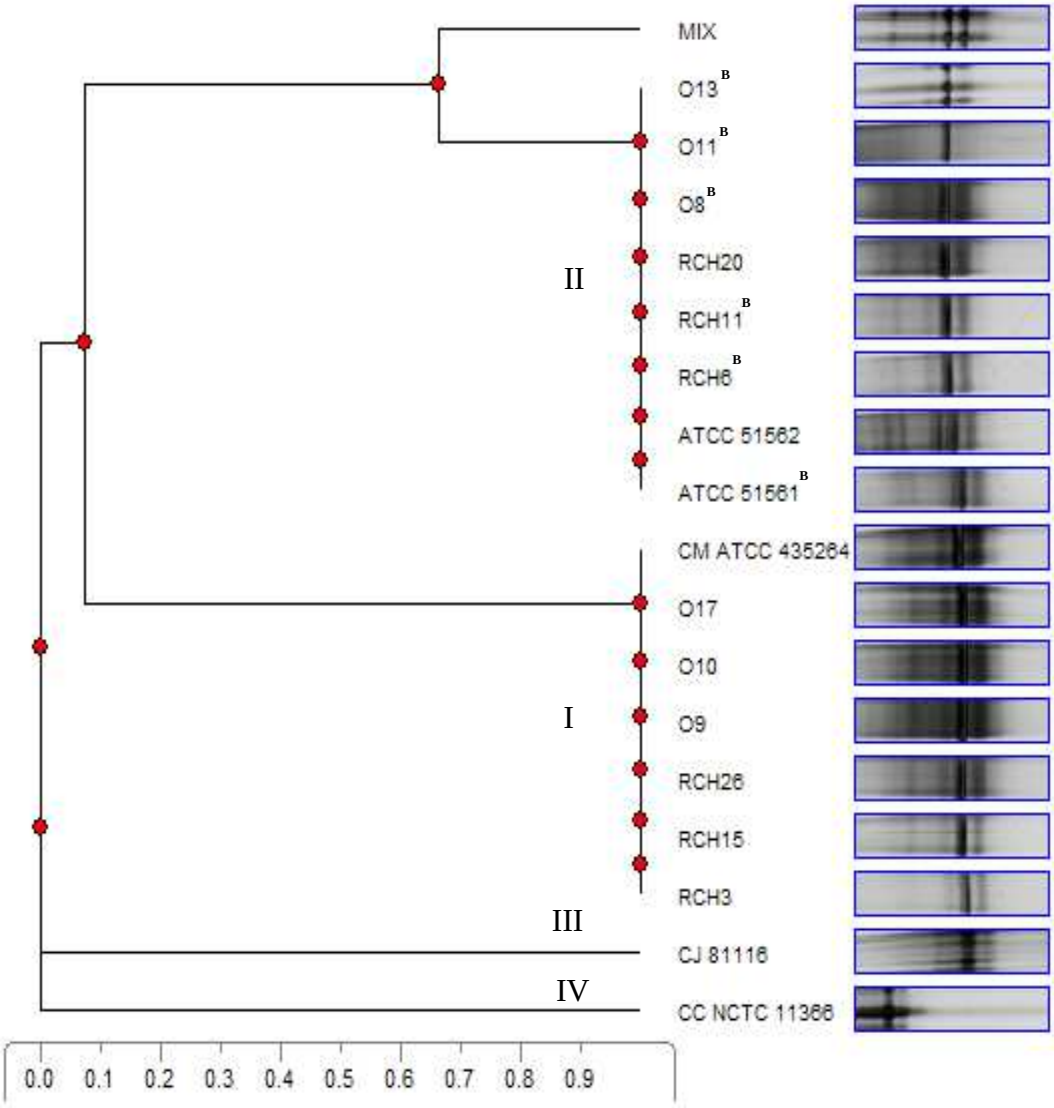


Figure 3.4: PCR-DGGE dendrograms using Muyzer primer set 2 targeting 16S rDNA in *C. concisus* and other *Campylobacter* reference strains. Dendrogram analyses were generated by Phoretix ID software for analysing PCR-DGGE gel results. ATCC 51561 and ATCC 51562: *C. concisus* reference strains. RCH: Royal Children's Hospital. O: oral isolates. CM: *C. mucosalis* (ATCC 43264), CJ: *C. jejuni* (81116), CC: *C. coli* (NCTC 11366) and *E. coli* (ATCC 25922), respectively. I-V are groups generated by the software. [B]: *C. concisus* isolates belonging to genomospecies B. Other *C. concisus* isolates are not marked as [B], belong to genomospecies A.

Table 3.1: *C. concisus* groups were generated by PCR-DGGE analysis using Muyzer primer sets I and II.

No.	Isolate	Genomospecies	Muyzer 1 (170 bp)	Muyzer 2 (562 bp)
1.	51562	A	I	II
2.	RCH3	A	II	I
3.	RCH15	A	I	I
4.	RCH20	A	I	II
5.	RCH26	A	II	I
6.	O9	A	I	I
7.	O10	A	I	I
8.	O17	A	II	I
9.	51561	B	II	II
10.	RCH6	B	II	II
11.	RCH11	B	II	II
12.	O8	B	II	II
13.	O11	B	II	II
14.	O13	B	II	II
15.	CM	NA	III	I
16.	CJ	NA	IV	III
17.	CC	NA	IV	IV
18.	EC	NA	V	NA

I, II, III and IV: generated groups by PCR-DGGE analysis using Muyzer primer sets 1 and 2. A & B: genomospecies. In each case, groups I and II were defined by the software. CM: *C. mucosalis* ATCC 43264. CJ: *C. jejuni* 81116. CC: *C. coli* NCTC 11366. EC: *E. coli* ATCC 25922. O; oral isolates. RCH: the Royal Children's Hospital isolates. NA: not applicable.

3.3.3 Detection of *cjaC* and investigation of the RCH3 fragment sequence location

3.3.3.1 Detection of *cjaC* (based on strain 13826)

Using primer set I, *cjaC* could only be amplified from genomospecies B isolates (Table 3.2 and Appendix III); however, primer set II designed to amplify 300 bp from the centre of *cjaC*, amplified *cjaC* from both genomospecies A and B, with the exception of six isolates belonging to genomospecies A (RCH3, RCH4, RCH5, RCH7, O12 and O17) (Table 3.2 and Appendix IV).

One reference strain (ATCC 51561) and one clinical isolate (RCH6), both from genomospecies B, were nominated as representatives for *cjaC* sequencing as their DNA could be amplified by both primer sets (I and II). The sequenced products obtained using primer set I are shown in Appendices V and VI. Alignment of 746 bp (nucleotide number 248 to 995) segments of *cjaC* of *C. concisus* ATCC 51561 and RCH6 to strain 13826 using Clone Manager software showed that the three sequences were highly similar with identities of 96% and 95%, respectively.

As primer set II was able to amplify *cjaC* from isolates belonging to genomospecies B and some isolates from genomospecies A, PCR product (295 bp) of *C. concisus* ATCC 51562 (from genomospecies B) was sequenced to confirm the identity of this PCR product (Appendix VII). The aligned sequence was 90% identical to the corresponding sequence of *C. concisus* 13826.

3.3.3.2 Investigating the RCH3 fragment sequence location

To investigate why the sequence of DNA fragment (containing one part of *cjaC* and part from shikimate 5-dehydrogenase genes) obtained from RCH3 did not exist in the genome of *C. concisus* 13826, primer sets III and IV were designed (based on RCH3 sequence) to

amplify the flanking region between *cjaC* and shikimate 5-dehydrogenase genes (Figure 3.2). Using primer set III to amplify 330 bp, PCR products were only obtained from genomospecies A isolates with the exception of O12 and O17 (Table 3.2 and Appendix VIII). Also, using primer set IV to amplify 270 bp of the same region (Figure 3.2 and Appendix IX), the PCR products were only obtained from genomospecies A isolates with the exception of RCH19 (Table 3.2).

The PCR sequenced products obtained with primer set III (330 bp) from the three genomospecies A isolates (ATCC 51562, RCH3 and RCH26) were 293 bp in length (Appendix X). Alignments of the three sequences showed 95% identity between these isolates with nucleotide sequence variation in at least 10 different locations (Appendix X). The variations in the sequences were very high when compared with *C. concisus* 13826 as the alignments with the corresponding *C. concisus* 13826 sequence revealed that the first 116 bp matched the first 119 bp of *cjaC* with an identity of ~88%; whereas the last 46 bp matched the end of the shikimate 5-dehydrogenase gene with an identity of ~85%.

To investigate presence of the *cjaC* gene (CCC13826_0963) and its adjacent (encoding a hypothetical protein (CCC13826_0962)) (Figure 3.2), primer sets V (514 bp) and VI (600 bp) were designed based on genome of *C. concisus* 13826 sequence. Using primer set V, PCR products were only obtained from 15.2% of *C. concisus* isolates (ATCC51561, RCH6, RCH11, O8 and O11) within genomospecies B. Furthermore, the PCR product of *C. concisus* ATCC 51561 was smaller in size (~330 bp) with less intensity than the other products (Table 3.2 and Appendix XI). Moreover, using primer set VI to cover an extended genomic region (600 bp) including a larger section of the gene encoding a hypothetical protein (CCC13826_0962) and a slightly larger section of *CjaC* (CCC13826_0963) than was obtained by primer set V, DNA of only two genomospecies B isolates (RCH6 and O11) were amplified. The amplified region from RCH6 was in the expected size, while the region

amplified from the DNA of O11 was larger in size (1700 bp) than predicted (Table 3.2 and Appendix XII). This larger PCR product was sequenced (Appendix XIII). Alignment with *C. concisus* 13826 DNA in the NCBI data-base showed homology (85%) to 244 bp of *cjaC* (CCC13826_0963) and the three adjacent genes encoding hypothetical proteins (CCC13826_0960, CCC13826_0961 and CCC13826_0962). The obtained sequence homologies over the three hypothetical protein genes are as follows; CCC13826_0962 was matched by 665 bp, CCC13826_0961 was matched by 143 bp and CCC13826_0960 was matched by 192 bp.

It is uncertain why primer set VI amplified a 600 bp PCR product from RCH6 and a 1700 bp PCR product from O11, while no PCR product was amplified from other isolates. It is unlikely that the forward primer did bind at the two DNA regions of the genome based on the PCR results as either only one PCR product or no PCR product was obtained from the isolates. It could be that, in the case of some isolates, the reverse primer bound at location CCC13826_0960 in the genome instead of CCC13826_0962. It would be difficult to investigate the effects of such variations in this region of *C. concisus* phenotype as the genes involved in this area do not encode for proteins with known functions.

3.3. 4 Detection and identification of *cjaA*, *dnaJ* and *zot*

The CjaA primer set amplified the DNA region from the majority of isolates (94%) except RCH15 and RCH23 (both from genomospecies A) (Table 3.3 and Appendix XIV). Using primer set CjaAT (Figure 3.1), the DNA region was amplified from all isolates with the exception of five isolates belonging to genomospecies A and four of these isolates were from faeces (Table 3.3 and Appendix XV).

Table 3.2: PCR amplification of DNA from the genome of *C. concisus* isolates using different primer sets targeting the regions of *cjaC* (CCC13826_0963) gene and its flanking regions.

Strain's name	Set I	Set II	Set III	Set IV	Set V	Set VI
ATCC 51561*	+	+	-	-	+*	-
RCH 6*	+	+	-	-	+	+
RCH 11*	+	+	-	-	+	-
O8*	+	+	-	-	+	-
O11*	+	+	-	-	+	+°
O13*	+	+	-	-	-	-
O16*	+	+	-	-	-	-
ATCC 51562	-	+	+	+	-	-
RCH 3	-	-	+	+	-	-
RCH 4	-	-	+	+	-	-
RCH 5	-	-	+	+	-	-
RCH 7	-	-	+	+	-	-
RCH 9	-	+	+	+	-	-
RCH 12	-	+	+	+	-	-
RCH 15	-	+	+	+	-	-
RCH 19	-	+	+	-	-	-
RCH 20	-	+	+	+	-	-
RCH 23	-	+	+	+	-	-
RCH 24	-	+	+	+	-	-
RCH 25	-	+	+	+	-	-
RCH 26	-	+	+	+	-	-
O1 - O7	-	+	+	+	-	-
O9 & O10	-	+	+	+	-	-
O12	-	-	-	+	-	-
O14	-	+	+	+	-	-
O17	-	-	-	+	-	-
C. mucosalis	-	-	-	-	-	-
C. jejuni	-	-	-	-	-	-
C. coli	-	-	-	-	-	-

C. mucosalis: ATCC 43264, *C. jejuni*: 81116, *C. coli*; NCTC 11366, O: oral isolates. RCH: the Royal Children's Hospital. NA: not applicable. *: small PCR product. °: large PCR product. *: genomospecies B.

Primer set DnaJ (covering 900 bp) could only amplify *dnaJ* from genomospecies B isolates. *C. jejuni* and *C. coli* genomic DNA also showed small nonspecific light bands (400 bp) (Appendix XVI). However, the second primer set (DnaJT, covering 304 bp) successfully amplified the gene from all *C. concisus* isolates except two from genomospecies A (O9 and O14) as shown in Appendix XVII and Table 3.3.

Using primer set ZoT, the *zot* gene could only be detected in 9% of isolates (RCH6, RCH11 and RCH26). The gene was amplified from these three isolates regardless of genomospecies as two were from genomospecies B (RCH6 and RCH11) and one belonged to genomospecies A (RCH26). The *zot* gene could not be detected in any oral isolates (Figure 3.5 and Table 3.3).

In summary, these results show that *C. concisus* isolates are genetically highly diverse. Primers sets I, II, III, IV, V and VI amplified the target DNA regions in the proportions at 21.2%, 81.8%, 72.7%, 72.7%, 15.2% and 6.1% respectively. Primers sets ZoT, CjaA, DnaJT and CjaAT amplified the target DNA regions in the proportions at 9%, 94%, 94% and 85% respectively. Primers sets I and DnaJ could be used for DNA detection of genomospecies B as they successfully detected all isolates from genomospecies B; whereas primers sets III and IV could be used for genomospecies A identification, since they detected 92.3% (24/26) and 96.2% (25/26) of isolates from genomospecies A.

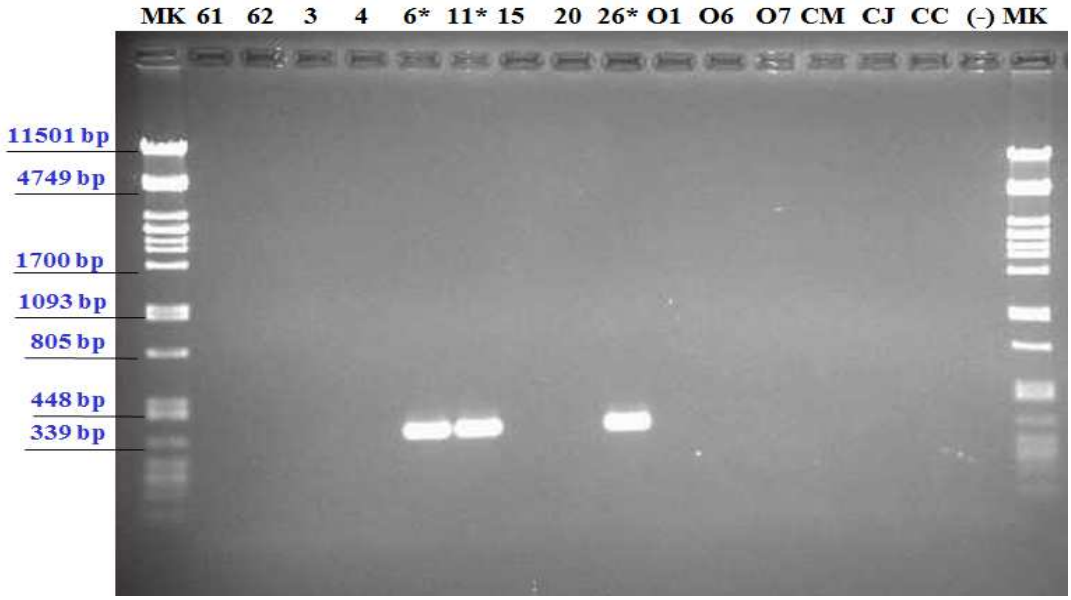


Figure 3.5: PCR amplification of *zot* in genomic DNA of *C. concisus* isolates using the ZoT primer set. PCR products were loaded onto a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. PCR product size was 355 bp. MK: lambda DNA marker. 61 & 62: *C. concisus* (ATCC 51561 &51562). 3, 4, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264). CJ: *C. jejuni* (81116). CC: *C. coli* (NCTC 11366,). O: oral isolates**.** *: genomospecies B.

Table 3.3: PCR amplification results using DNA obtained from *C. concisus* isolates with different primer sets for the regions of *cjaA*, *dnaJ* and *zot*.

Strain's name	CjaA	CjaAT	DnaJ	DnaJT	ZOT
ATCC 51561*	+	+	+	+	-
RCH 6*	+	+	+	+	+
RCH 11*	+	+	+	+	+
O8*	+	+	+	+	-
O11*	+	+	+	+	-
O13*	+	+	+	+	-
O16*	+	+	+	+	-
ATCC 51562	+	-	-	+	-
RCH 3	+	+	-	+	-
RCH 4	+	+	-	+	-
RCH 5	+	+	-	+	-
RCH 7	+	+	-	+	-
RCH 9	+	+	-	+	-
RCH 12	+	+	-	+	-
RCH 15	-	-	-	+	-
RCH 19	+	+	-	+	-
RCH 20	+	-	-	+	-
RCH 23	-	+	-	+	-
RCH 24	+	+	-	+	-
RCH 25	+	+	-	+	-
RCH 26	+	-	-	+	+
O1 - O7	+	+	-	+	-
O9	+	+	-	-	-
O10	+	+	-	+	-
O12	+	+	-	+	-
O14	+	+	-	-	-
O17	+	-	-	+	-
C. mucosalis	-	-	-	-	-
C. jejuni	-	-	-	-	-
C. coli	-	-	-	-	-

RCH: the Royal Children's Hospital isolates. ATCC: American Type Culture Collection strains. *C. mucosalis*: ATCC 43264. *C. jejuni*: 81116. *C. coli*: NCTC 11366. O: *C. concisus* oral isolates. +: DNA amplification product was obtained, -: No DNA amplification product was obtained. NA: not applicable. *: genomospecies B.

3.3.5 Bioinformatics analysis following the recent publications of *C. concisus* sequence

Based on primer sets MUC1 and CON1 or CON2, all fully sequenced *C. concisus* strains available on the data base (section 1.9.4) belonged to genomospecies B, with the exception of strain ATCC 51562 which belonged to genomospecies A. Searching through the NCBI data-base site showed that genes all examined in this study (*cjaC*, *cjaA* and *dnaJ*) with the exception of *zot*, were present in all the sequenced strains.

CjaC

Alignment of the *cjaC* sequence of the published *C. concisus* strains and RCH6 (746 bp) revealed an identity of ≥96% between all strains with the exception of *C. concisus* strain ATCC 51562, which showed only identity of 88%. Each strain showed a unique nucleotide sequence of this gene. All strains showed changes in nucleotide sequences at ≤42 bp positions; whereas, in *C. concisus* strain ATCC 51562 the changes were at 129 positions.

The NCBI data-base site, used to investigate the impacts of nucleotide changes on amino acids sequence differences in CjaC, revealed an identity of 99% for all strains with the exception of strain ATCC 51562 which had identity of only 95%. Amino acids substitutions occurred at 1-5 positions in each strain with the exception of strain ATCC 51562, which showed changes at 15 amino acid positions. Excluding strain ATCC 51562, five amino acid substitutions were located at positions 31, 42, 49, 133, 161, 205 and 234.

Neighbour-joining dendrogram analysis showed that *cjaC* gene and corresponding amino acids sequences could classify the strains into two main groups (I and II). All the strains were allocated into group II excluding ATCC 51562, which fitted into group I (Figures 3.6 and 3.7).

DnaJ

Analysis of *dnaJ* sequence and corresponding amino acid sequences revealed that all the strains had different nucleotide sequences. Identity scores of the gene ranged from 95-96% (mismatching in ≤61 bp) with the exception of ATCC strain 51562, which had a score of 86% (mismatching in 160 bp). Amino acid sequences of all strains were 99% identical with the exception of ATCC 51562, which was 95% identical to the other strains. This strain had changes at 20 amino acid positions (similarity at 11 and mismatching at 9). In contrast, the other strains had changes at 3-5 amino acid positions and mismatching was at one location. The corresponding dendrograms also showed that both DNA and amino acid sequences could classify the strains into two main groups (I and II) (Figures 3.8 and 3.9). The majority of the strains were allocated into group II excluding strain ATCC 51562, which fitted in group I.

CjaA

Alignment of the DNA sequences of *cjaA* in the sequenced genomes of the *C. concisus* with the sequence of *C. concisus* 13826 showed that all strains have different nucleotide sequence. For all the strains, the identity was ≥95% (the changes were at ≤40 bp positions), with the exception of *C. concisus* ATCC 51562 that had an identity score of 88% (the changes included 104 bp). A neighbour-joining dendrogram analysis divided the strains into two main groups, with *C. concisus* strain ATCC 51562 in dendrogram group I and all other strains in group II (Figure 3.10).

The alignments of the amino acid sequences translated from *cjaA* nucleotide sequences showed that the identities were at ≥98% in the strains excluding *C. concisus* ATCC 51562, which was 95% identical. Neighbour-joining dendrogram analysis also divided *C. concisus* ATCC 51562 into an individual group I while other strains fell into group II (Figure 3.11).

Summary of sequencing analysis

Based on primers designed from the only sequence that was available in the NCBI data base at the commencement of this study (NCBI 13826), it was thought that the studied putative virulence genes might not be possessed by some *C. concisus* isolates. Although two primer sets designed based on strain 13826 sequence to amplify *cjaC* (I and II) and *dnaJ* (DnaJ and DnaJT), these genes could not be detected in all the studied *C. concisus* isolates. After the sequences of seven more strains became available, in particular ATCC 51562 (genomospecies A), all genes except *zot* were found to be present in all sequenced stains. The data obtained from amplification of using the designed primer sets suggested that there was considerable sequence variation between the genomospecies A and B and this was confirmed by neighbour-joining phylogenetic analysis of the sequences that later became available. The central region of *cjaC, cjaA* and *dnaJ* appears to be more conserved than the 5` and 3` extremities of the genes as all the primer sets (II, DnaJT and CjaAT), designed to target the centre of these genes were able to amplify them from most of *C. concisus* tested isolates.

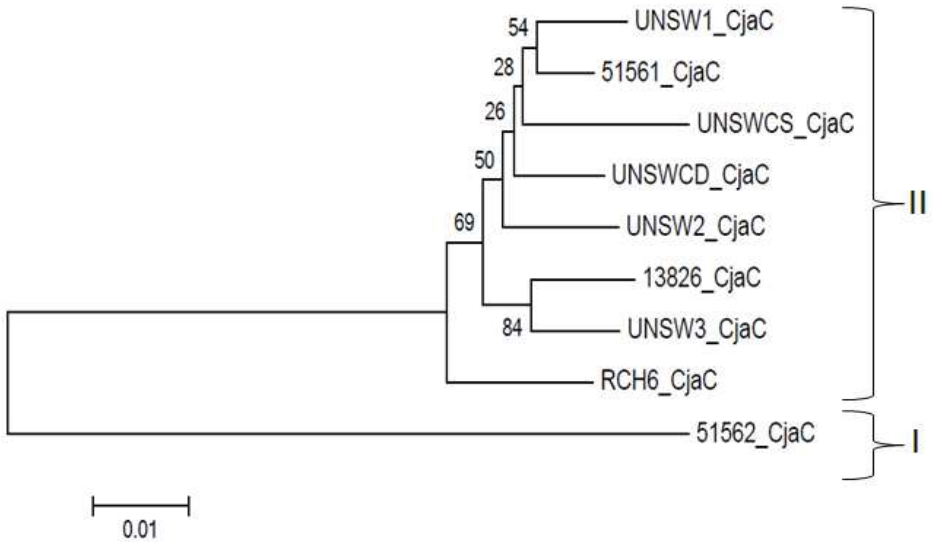


Figure 3.6: Neighbour-joining dendrogram for *cjaC* sequences obtained from nine *C. concisus* strains. I and II are the main groups of *C. concisus* strains that can be generated by neighbour-joining dendrogram. Group I includes the *C. concisus* genomospecies A strain 51562, while group II includes genomospecies B strains.

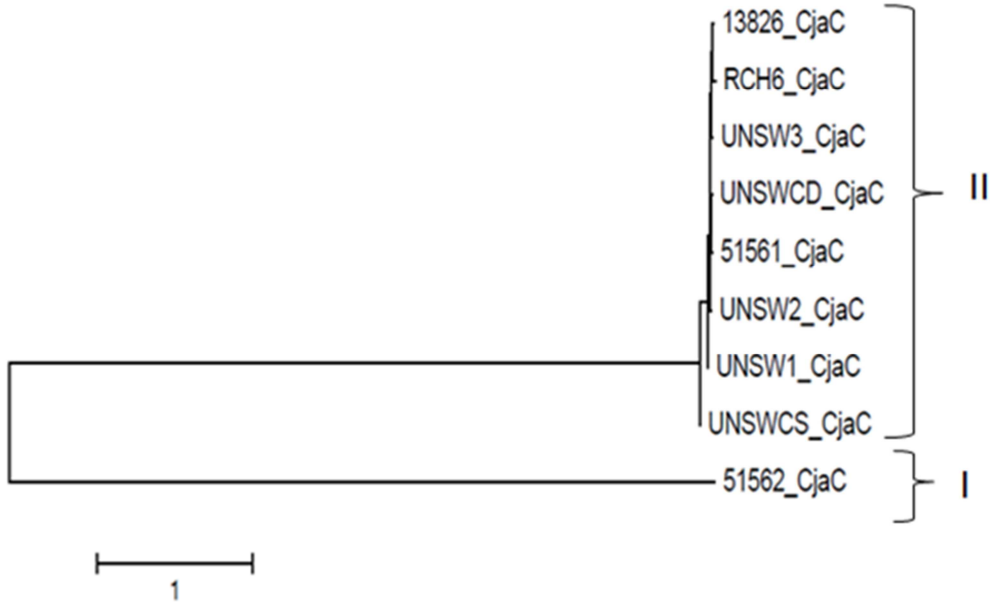


Figure 3.7: Neighbour-joining dendrogram for the amino acid sequences translated from the *cjaC* gene obtained from the data base of eight sequenced *C. concisus* strains and RCH6. I and II are the main groups of *C. concisus* strains that can be generated by neighbour-joining dendrogram. Group I includes the *C. concisus* genomospecies A strain (ATCC 51562), while group II includes genomospecies B strains. The whole genome sequence of strain 13826 is complete and available in the NCBI data-base site. Strains 51561, 51562, UNSW1, UNSW2, UNSW3, UNSWCS and UNSWCD are Whole-genome shotgun contigs (WGS) available in the NCBI data-base site. Strain RCH6 gene was sequenced in this study.

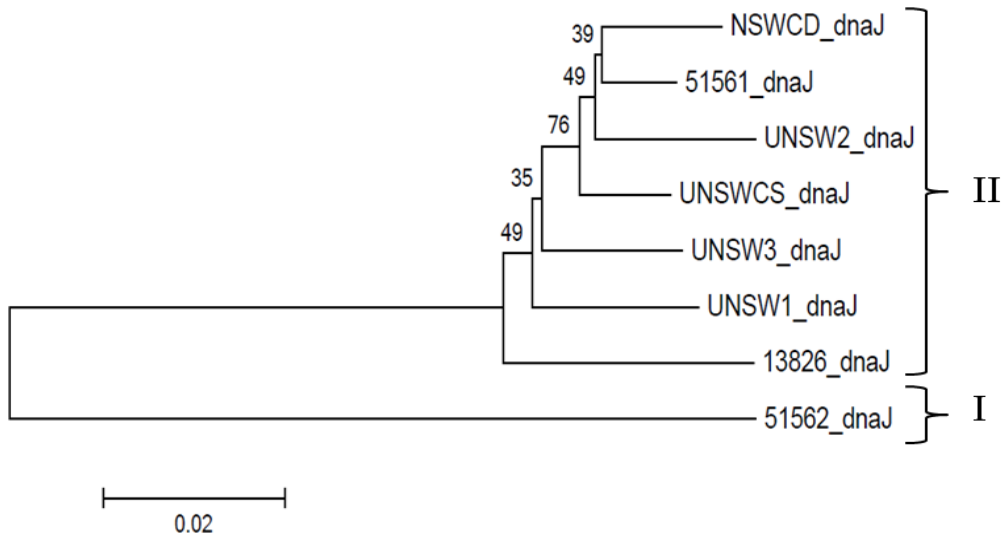


Figure 3.8: Neighbour-joining dendrograms for *dnaJ* sequences obtained from eight sequenced *C. concisus* strains available in the NCBI data-base site. I and II are the main groups of *C. concisus* strains that can be generated by neighbour-joining dendrogram. Group I includes the *C. concisus* genomospecies A strain 51562, while groups II includes genomospecies B strains.

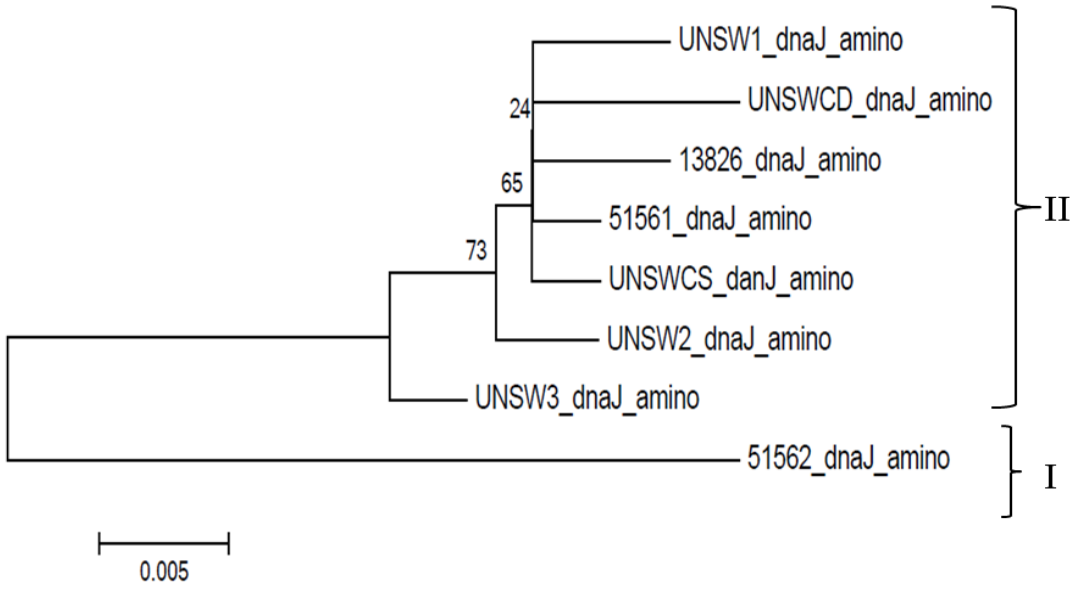


Figure 3.9: Neighbour-joining dendrogram for the amino acid sequences translated from the *dnaJ* gene obtained from eight sequenced *C. concisus* strains available in the NCBI data-base site. I and II are the main groups of *C. concisus* strains that could be generated by neighbour-joining dendrogram. Group I includes the *C. concisus* genomospecies A strain 51562, while group II includes genomospecies B strains. The whole genome sequencing of strain 13826 is complete and available in the NCBI data-base site. Strains 51561, 51562, UNSW1, UNSW2, UNSW3, UNSWCS and UNSWCD are WGS available in the NCBI data-base site.

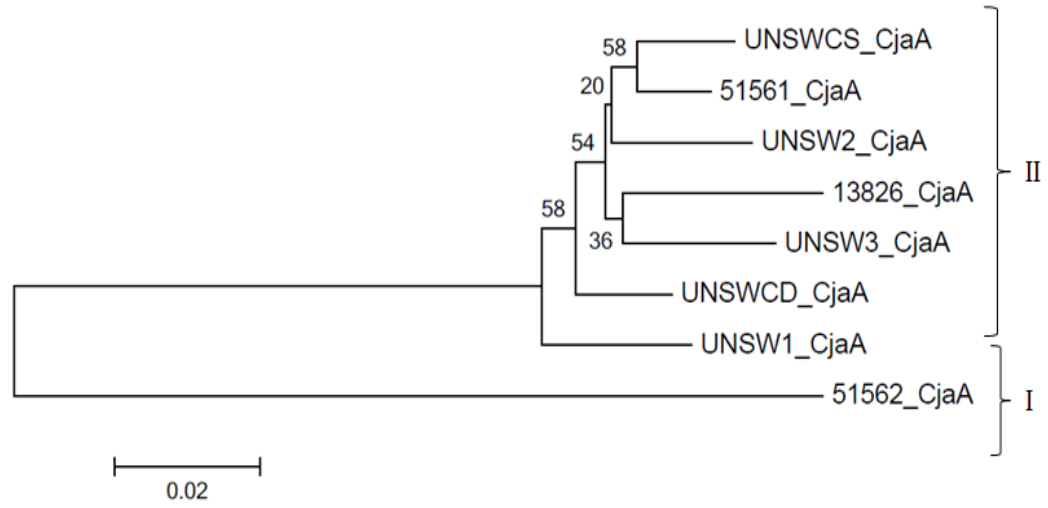


Figure 3.10: Neighbour-joining dendrogram for *cjaA* sequences obtained from eight *C. concisus* strains available in the NCBI data-base site. I and II are the main groups of *C. concisus* strains that could be generated by neighbour-joining a dendrogram. Group I comprises the *C. concisus* genomospecies A strain 51562 while group II includes genomospecies B strains.

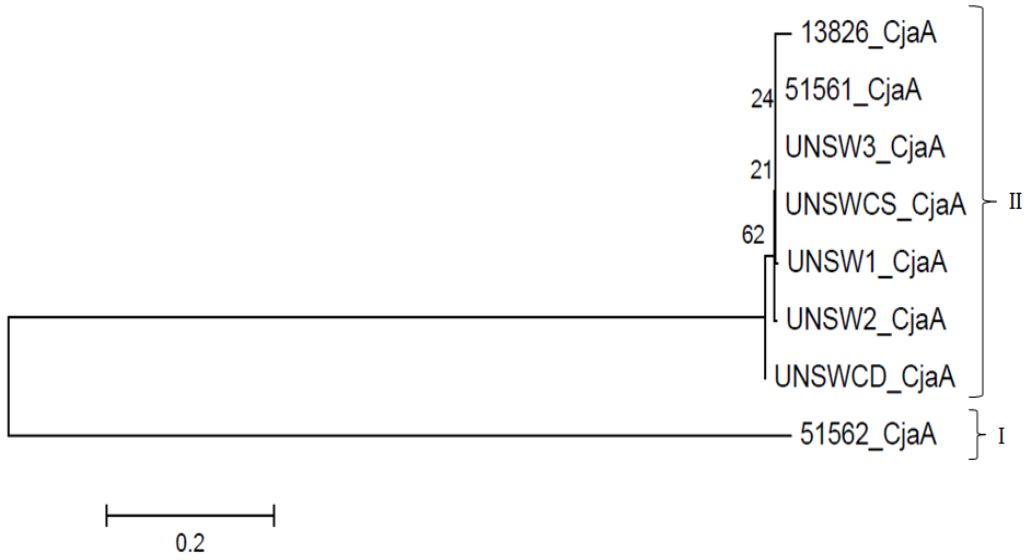


Figure 3.11: Neighbour-joining dendrogram based on the amino acid sequences translated from *cjaA* of sequenced *C. concisus* strains available in the NCBI data-base site. The strains used were whole genome sequenced including strain 13826 which is a completed whole genome sequence while 51561, 51562, UNSW1, UNSW2, UNSW3, UNSWCS and UNSWCD (available in the NCBI data-base site as WGS). I and II are the main groups of *C. concisus* strains that can be generated by neighbour-joining dendrogram. Group I comprises the *C. concisus* genomospecies A strain 51562 while group II includes genomospecies B strains.

3.4 Discussion

PCR-DGGE analysis and PCR amplification of several putative virulence genes were applied to a panel of *C. concisus* isolates obtained from two different sites (the oral cavity and intestine) to investigate their genetic diversity. The study also investigated the prevalence of these genes and their sequence variation in genomospecies A and B using the published genomes of *C. concisus* strains. The results of the current study consistently enhanced our awareness of the large genetic heterogeneity of *C. concisus* isolates obtained from faecal (diarrhoeic patients) and oral (healthy volunteers) specimens.

In the confirmation of the identity of *C. concisus* isolates used in the study, all the isolates were amplified with either MUC1 and CON1 or MUC1 and CON2, which agreed with Istivan *et al.* (2004) findings. It is noteworthy that for one *C. concisus* isolate (RCH20) no product was amplified using Pcisus primer (5 & 6), which has not been reported before.

DGGE typing of *C. concisus*

The DNA of all *C. concisus* tested isolates was amplified using both Muyzer primer sets (1 and 2) to amplify 16S rDNA; thus, these primer sets could be used to detect *C. concisus* in mixed DNA extracted from clinical samples. The data obtained in this study from DGGE analysis suggested that using Muyzer primer set 1 with *C. concisus* isolates is more accurate than using Muyzer primer set 2 as *C. mucosalis* could not be distinguished from *C. concisus* group II isolates generated by DGGE analysis using Muyzer primer set 2. It is noteworthy that three *C. concisus* isolates possessing *zot* were assigned to group II by DGGE using Muyzer primer set 1.

Based on the results from the DGGE gel dendrogram analysis using both Muyzer primer sets, all genomospecies B isolates were assigned into one group, confirming the findings of Istivan *et al.* (2004). However, genomospecies A isolates fell into the two groups (I and II) on DGGE analysis, which is inconsistent with previous the genomospecies typing based on 23S

rDNA (Istivan *et al.* 2004). *C. concisus* genomospecies A isolates assigned to group I by DGGE using Muyzer primer set 1 were different from those allocated to group I using Muyzer primer set 2. The possible reason for the difference is that in the DGGE technique, regions of the 16S rDNA were used, while Istivan *et al.* (2004) used 23S rDNA. Thus, the results of genotyping of *C. concisus* isolates possibly depend on the DNA region used.

A previous study found that PCR-DGGE was also able to assign three *C. concisus* reference strains into two DGGE groups (Petersen *et al.* 2007); however, another study found all *C. concisus* isolates were assigned in one group using the same technique (Cornelius *et al.* 2012). Other genetic typing methods such as MLST and RAPD also assigned *C. concisus* isolates in two main groups (Ismail *et al.* 2012; Matsheka *et al.* 2006; Miller *et al.* 2012). However, it is unclear whether the groups generated by these techniques allocate *C. concisus* isolates in the same genomospecies A and B.

Putative virulence genes in *C. concisus* isolates

The putative virulence genes (*cjaC, cjaA, dnaJ* and *zot*) were amplified in 81.8%, 85%, 94% and 9%, respectively of *C. concisus* isolates using primer sets targeting the central region of these genes. However, based on the bioinformatics analysis performed in this study using the published genomes of *C. concisus* strains, it was found that all of these genes were present with an exception of *zot*, which was only detected in 37.5% (3/8) of the isolates. Therefore, *cjaC, cjaA* and *dnaJ* are probably possessed by the majority of *C. concisus* isolates, while *zot* are carried by the minority of the isolates.

In this study, the detection rates of *cjaC, cjaA* and *dnaJ* were improved with primer sets that amplified the centre of these genes, while Kaakoush *et al.* (2011a) could not show any difference when they used two primer sets to detect *zot* from *C. concisus* isolates that were designed to amplify 979 bp and 790 bp of the gene (1125 bp). The possible reasons for these

discrepancies are that the sequences of these genes are heterogeneous within *C. concisus* isolates or that some of the genes are not possessed by all of the isolates.

Conflicting results were shown also in detection of genes by PCR in *C. jejuni* and *C. coli. CjaC* and *cjaA* could not be amplified from 33.3% of *C. jejuni* clinical isolates (Pawelec *et al.* 2000). It has also been recognised that specific primers that were designed for *cjaC* based on the nucleotide sequence of *C. coli* do not amplify the gene from other species like *C. jejuni* (Garvis *et al.* 1996; Pawelec *et al.* 1998; Pawelec *et al.* 2000). In *C. jejuni,* it was reported that there is genotypic variation without any phenotypic change, including the CjaC protein function and the specific antibody reaction (Pawelec *et al.* 2000). The change in the gene sequence in *C. concisus* also may not result in phenotypic changes such as morphological characteristics or bacterial growth on HBA.

Moreover, the detection rate of *dnaJ* was 64% and 11% in *C. jejuni* and *C. coli* clinical isolates respectively (Hamidian *et al.* 2011), while in another study, the detection rate of the gene in *C. jejuni* isolates by PCR was 100% (Chansiripornchai *et al.* 2009).
The detection rates of *cjaC, cjaA* and *dnaJ* in the faecal isolates were 76%, 76% and 100%, and in the oral isolates they were 88%, 94% and 88%, respectively. There was no significant difference in the detection rates of these genes between the faecal and oral isolates. However, the *zot* gene was only detected in faecal isolates.

There are conflicting reports on the prevalence of *zot* among *C. concisus* isolates from faeces. Excluding the oral isolates in this study, *zot* detection in faecal isolates was 20% of faecal isolates, which was similar to a previous study finding (22%, 3/17) that also investigated the gene in faecal isolates (Kalischuk *et al.* 2011). However, Kaakoush *et al.* (2011b) could not detect *zot* in any of *C. concisus* (4 isolates obtained from intestinal biopsies and 3 faecal

isolates). These results could be explained if *zot* displays sequence variation or is possessed by minority of *C. concisus* isolates.

Location of RCH3 fragment in *C. concisus* genome

In this study, primer sets III and IV were designed based on DNA fragment obtained from RCH3 (determined to be a genomospecies A strain) to amplify parts of *cjaC* and the shikimate 5-dehydrogenase genes (Istivan, *unpublished* data). These primer sets detected the majority of genomospecies A isolates, but none of genomospecies B isolates. Based on strain 13826 genome sequence (a genomospecies B strain) the gene adjacent to the *cjaC* gene (CCC13826_0963) encodes a hypothetical protein (CCC13826_0962), while the shikimate 5-dehydrogenase gene (CCC13826_0229) is apart from *cjaC*. Therefore primer set V was designed to amplify parts of *cjaC* and the gene encoding the hypothetical protein gene (CCC13826_0962). Primer set V could detect the majority of genomospecies B isolates (71.4%), while none of genomospecies A isolates were amplified. It was initially thought that either *cjaC* or the shikimate 5-dehydrogenase gene might be located in different parts of the genome of genomospecies A isolates compared with B isolates. In addition, it can be suggested that genomospecies A strains might possess a second copy of *cjaC* since the total number of genes in the *C. concisus* UNSWCD genome was less than the total number of genes in the genome of *C. concisus* 13826 (Kaakoush *et al.* 2011a). However, the whole genome sequence of the *C. concisus* strains revealed that all strains possessed *cjaC* and the shikimate 5-dehydrogenase genes in a non-contiguous organisation, except for *C. concisus* ATCC 51562, which has the genes in an adjacent location in the genome (Deshpande *et al.* 2013). The order of these genes varied, even within genomospecies B. In the genome of *C. concisus* ATCC 51561, UNSW2, UNSW3, UNSWCS and UNSWCD, the genes were in different contigs, but in *C. concisus* strain UNSW1 they were present on the same contig with another gene in between (Deshpande *et al.* 2013). The data suggested that the shikimate 5-

dehydrogenase is apart from *cjaC* in the genome of genomospecies B isolates while in the majority of genomospecies A isolates, the genes are adjacent. On other hand, it seemed from the data obtained in this study that the location of *cjaA* is similar in most of *C. concisus* isolates; as the DNA region that included *cjaA* and its adjacent gene (glutamine transport ATP-binding protein GlnQ) was detected in the majority of the tested isolates (94%, 31/33) using primer set CjaA (based on strain 13826 sequence).

Diversity of putative virulence genes in published genomes of *C. concisus*

Neighbour-joining phylogenetic analysis for sequenced *C. concisus* available on the data base for the *dnaJ, cjaA* and *cjaC* DNA revealed none of these genes is identical between the strains and all of these genes are highly heterogeneous within genomospecies A and B. The phylogenetic analysis for the sequence of translated amino acids of these genes also showed diversity between genomospecies A and B strains, but this diversity is less within same genomospecies isolates. The diversity between the two genomospecies A and B is vast. Both phylogenetic analysis of DNA and the corresponding amino acids presented two main groups (I and II), which was consistent with phylogenetic analysis for 23S rDNA and the peptidoglycan biosynthesis pathway genes of these isolates (Deshpande *et al.* 2013; Istivan *et al.* 2004). However, the phylogenetic analysis for the 16S rDNA gene allocated these isolates into three groups (Deshpande *et al.* 2013), which contradicted DGGE results and genomospecies (A and B). These outcomes could be because the whole of the 16S rDNA gene (1550 bp) was used in the phylogenetic analysis, whereas DGGE analysis targeted ~920 bp of the 16S rDNA gene. The phylogenetic analysis based on 23S rDNA agreed with the gene groups investigated in this study, whereas analysis based on the 16S rDNA gene generated unique groups. Now that whole genome sequencing is available, there will be a demand for additional whole genome sequencing of *C. concisus* genomospecies A isolates as only one strain has been sequenced (ATCC 51562).

Similar results have been reported for other members of *Campylobacterales*. The most genetically divergent member is *Helicobacter pylori* based on the whole genome sequencing (Bjrkholm *et al.* 2001; Marshall *et al.* 1998). It was reported that PCR may not amplify DNA from all *H. pylori* isolates as the sequences of several genes such as flagellin (*flaA* and *flaB*) and urease (*ureA*, *ureB* and *ureC*) varied between strains (Marshall *et al.* 1998).

In summary, this study revealed that the *cjaC, cjaA* and *dnaJ* genes exist in the majority of *C. concisus* isolates. However, *zot* was found in only 20%-25% of *C. concisus* isolates. DNA sequence variations possibly led to PCR amplification failure to detect virulence genes in some *C. concisus* isolates. The variations are vast between genomospecies A and B, and even in corresponding amino acids, although a lesser extent than DNA sequence variations. PCR-DGGE based on the 16S rDNA gene using Muyzer primer sets, clustered *C. concisus* isolates into two distinguishable groups. The results of PCR assays suggested that although the detection of putative virulence genes could be improved by the second designed primer set designed based on strain 13826 to amplify sequences in the centre of the gene, there were some specific genomospecies A stains that cannot be detected using either primer set. It is suggested that targeting the central part of putative virulence genes might improve the detection, but this needs more confirmations. In using of the two methods (PCR-DGGE and PCR amplification), no differences between *C. concisus* isolates were recognised between being oral or faecal with the exception of the detection of *zot.*

Future directions

Based on 23S rDNA, genomospecies A was the most prevalent in both oral and faecal isolates, with prevalence of 75% and 82.4%, respectively, which was not statistically significant. Further studies are required to evaluate the virulence of *C. concisus* clinical isolates within each genomospecies or group. The DGGE technique could be used to separate *C. concisus* isolates to groups, but more studies are required to determine whether detection

of *C. concisus* groups in mixed bacterial DNA is feasible and to investigate any difference in the virulence state of the generated groups. Sequencing *cjaC*, *dnaJ* and *cjaA* of other *C. concisus* isolates, particularly from genomospecies A to be used in the phylogenetic analysis to support this study findings. In addition, whole genome sequencing for more genomospecies A strains is required as only one strain has been sequenced up to date. Performing protein studies on the studied genes would be essential to investigate if the protein properties are similar within the two genomospecies.

Chapter 4 : The interaction between *C. concisus* and host epithelial cells *in vitro*

4.1 Introduction

Several studies have reported that pathogenic *Campylobacter* spp. such as *C. jejuni* are able to adhere to and invade epithelial cells *in vitro* and this process is dependent on the expression of several virulence factors (Bereswill *et al.* 2002; Ketley 1997; Russell *et al.* 1993). The association of *C. concisus* with human diseases is still unclear; hence, investigating the possible virulence factors in *C. concisus in vitro* could be an initial step to characterise the potential role of *C. concisus* in human infections.

C. concisus strains differ in their ability to invade intestinal epithelial cell lines and *C. concisus* UNSWCD was the most invasive examined in one study (Man *et al.* 2010a). Interestingly, faecal and oral *C. concisus* isolates obtained from healthy individuals were able to attach, but were unable to invade human cell lines, unlike isolates obtained from biopsies and the oral cavity of CD patients (Ismail *et al.* 2012; Kaakoush *et al.* 2011b). Given that *C. concisus* isolates are genetically heterogeneous; a specific group is possibly involved in human infections.

In *in vitro* studies of invasion, the highest reported invasion level of *C. concisus* was reported at a minimum of six hours and it was associated with cytoskeletal change in the epithelial cells, and an increase in the inflammatory factors TNF-α and IFN-γ (Kaakoush *et al.* 2011b). The elevation of these inflammatory factors has also been previously noted as a part of the clinical features of CD patients (Fuss *et al.* 1996; Murch *et al.* 1993).

This part of the study aimed to:

I. Establish a *C. concisus* growth curve to assess the conditions for optimal growth and to establish a reproducible recovery procedure from broth cultures.

II. Assess and compare the capacity for adhesion and invasion of INT407 cells of a panel of *C. concisus* faecal and oral isolates belonging to genomospecies A and B.

III. Investigate the expression of putative virulence genes (*cjaC, cjaA, dnaJ* and *zot*) by *C. concisus* isolates (detected positive for these genes in chapter 3) grown in different synthetic media and in INT407 cells to possibly assess virulence state as there are no published reports on the expression of these putative virulence genes from *C. concisus.*

4.2 Materials and Methods

4.2.1 Growth curve

C. concisus strain ATCC 51562 was selected for performing the growth curve study. The strain was grown on HBA for 48 h as described in section 2.4.2. Five to seven colonies were suspended in 1 ml of brain heart infusion broth (BHI) and the OD_{600} value was adjusted at 0.1 (0.5 McFarland) using an Eppendorf BioPhotometer. From the suspension, 100 µl was inoculated into each of ten tubes containing 0.9 m1 of BHI. The tubes were then incubated under standard growth conditions as described in section 2.4.2. The OD_{600} value of the growing culture was measured and recorded at 0 h and 4 h (to detect the lag phase) and was then measured every 12 h for 9 days. The bacterial viability (CFU) was also determined at the same time as the OD_{600} reading. To assess the CFU value at each time point, serial dilutions were performed by spreading 100 µl aliquots of bacterial cultures onto a HBA plate as a lawn, followed by incubation under the specific growth conditions indicated in section 2.4.2 for *C. concisus*. Three independent experiments were performed in duplicate.

In addition during the performance of the growth curve, *C. concisus* growth was tested on Columbia agar base (CA) without the addition of blood. *C. concisus* growth on CA was examined by the preparation of lawn culture from fresh bacterial growth on HBA, and inoculated onto CA.

4.2.2 Invasion and adhesion assays

INT407 cells are a derivative of cells obtained from a malignant tumour in the small intestine of a human embryo (Henle *et al.* 1957). These cells can maintain the morphological and immunological characters of intestinal crypt cells and grow *in vitro* as a monolayer with formation of tight junctions (Grönroos *et al.* 1998; Henle *et al.* 1957; Nickerson *et al.* 2001). Thus, it was proposed that INT407 cells would be an appropriate model for *C. concisus* infection and for investigating differences in adhesion and invasion between isolates from genomospecies A & B.

Bacterial isolates and strains used in the adhesion and invasion assays were: 10 *C. concisus* isolates and two *Campylobacter* reference strains (*C. jejuni* 81116 and *C. coli* NCTC 11366). Five *C. concisus* isolates were from genomospecies A (ATCC 51562, RCH3, RCH26, O10 and O12) and same number of *C. concisus* isolates was from genomospecies B (ATCC 51561, RCH6, RCH11, O11 and O13). *C. concisus* isolates RCH6, RCH11 and RCH26 possess the *zot* gene (section 3.3.4). Invasion and adhesion assays were performed as described in section 2.11.5.

A *C. concisus* isolate with an invasion index ≥1 (section 2.11.5) was considered to be an invasive as described by Ismail *et al.* (2012), based on a previous study that showed that *C. jejuni* strains with an invasion index of ≥1 had clinical symptoms in infected pigs, similar to human symptoms (Ismail *et al.* 2012; Larson *et al.* 2008).

4.2.2 Gene expression studies

Initially, levels of gene expression were determined for *C. concisus* ATCC 51561 (three independent experiments in duplicate). For the three remaining *C. concisus* isolates (ATCC 51562, RCH6 and RCH26), one experiment was performed. The targeted genes (*cjaC, cjaA, dnaJ* and *zot*) were amplified with primer sets II, CjaAT, DnaJT and ZoT (Table 2.2).

Relative gene expression was determined using semi-quantitative SYBR green assay, using *atpA* (ATP synthesis F1 complex alpha subunit) as a housekeeping gene. Based on the sequence of *C. concisus* strain 13826, the sequence of the AtpA forward primer was 5`-TGGCGCTATGGACTACACAA-3` and the sequence for the AtpA reverse was 5`-TCAAAGATCCAGCGCCTAGT-3` to amplify a 300 bp product.

RNA was extracted from *C. concisus* grown on HBA and CA. In addition, *C. concisus* RNA was extracted from bacterial cells maintained in INT407 cells growing in DMEM/FBS (DMEM+INT407) and DMEM/FBS (DMEM).

To extract *C. concisus* RNA, bacterial suspensions from 48 h old cultures on HBA or CA were prepared in Columbia broth and the OD_{600} values were adjusted to 0.1. The bacterial pellets were collected from 10 ml of the bacterial suspension, washed twice by re-suspension in PBS and centrifugation. The cleaned bacterial pellets were then subjected for RNA extraction as described in section 2.12.3.

To extract RNA from bacterial cells maintained with INT407 cells, *C. concisus* cells were introduced to DMEM+INT407 and DMEM as explained in the adhesion and invasion assays (section 2.11.5). After the incubation for 6 h, *C. concisus* cells were obtained by collecting (from 20 wells of the plate for each bacterial isolate) the medium from both plates containing DMEM+INT407 and DMEM. The medium containing *C. concisus* cells was centrifuged at 5000 x *g* for 4 min. The supernatant was discarded and the pellet was used for RNA extraction as described in section 2.12.3. In the same way DMEM of wells containing

INT407 cells (no bacterial cells were added) was collected as above to be used as control negative template (to insure the bacterial primers do not attach and amplify cDNA that may be synthesised from INT407 cells).

cDNA synthesis was described in section 2.12.4. A standard curve was generated by performing serial dilutions from genomic DNA. Three dilutions (descending by 50 folds) were generated starting from 0.2, 0.004 and 0.00008 ng/µl. In each RT-PCR reaction, 5 µl were used. RT-PCR conditions were described in section 2.12.5.

4.2.4 Determination of the lowest number of bacterial cells yielding sufficient RNA for cDNA synthesis.

In order to determine the lowest number of bacterial cells from which sufficient RNA for cDNA synthesis could be extracted, bacterial suspensions of *C. concisus* ATCC 51562 were prepared as following 10^9, 10^7, 10^5 and 10^3 CFU. Initially, the bacterial concentration was by adjusting by OD_{600} value to 0.1, which had been previously determined to contain 10^8 CFU.

4.2.6 Data analysis

The mean and standard deviation (SD) of datasets, and growth curves were plotted using Microsoft Excel. Statistical analysis was performed using GraphPad Prism 6 software (USA) using unpaired T-test where appropriate to calculate *P* values for invasion/adhesion assays and gene expression study. A value of $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Growth curve

Based on the OD_{600} reading, mid-log phase was at 2.5 days (OD_{600}: 0.243 ~log 8.91 CFU) as shown in Figures 4.1.

In addition, it was found that *C. concisus* cells from either fresh culture or frozen stock were able to grow on fresh CA, which has not been reported previously. The colony size and shape on CA were similar to those of colonies grown on HBA (Figure 4.2).

4.3.2 Adhesion and invasion assays

The adherence of *C. concisus* isolates (ATCC 51561, O12 and O11) and *C. coli* NCTC 11366 to INT407 cells was significantly higher than the reference strain (*C. jejuni* 81116) (P <0.05) (Figure 4.3). The adherence value of the remaining isolates (ATCC 51562, RCH3, RCH6, RCH11, RCH26, O10 and O13) was similar to *C. jejuni* 81116 (P >0.05). P value represents the calculation of statistical significance between two or more compared values, P <0.05 refers to significant difference between the compared values while P >0.05 refers to no significant difference between the compared values.

When the invasion capability of the above isolates was assessed, the isolates were different in their ability to invade INT407 cells. *C. concisus* ATCC 51561 and O12, and *C. coli* NCTC 11366 demonstrated significantly higher invasion rates than *C. jejuni* 81116 (P <0.05), while RCH6 and RCH26 had significantly lower invasion rates than *C. jejuni* 81116 (P <0.05) (Figure 4.4).

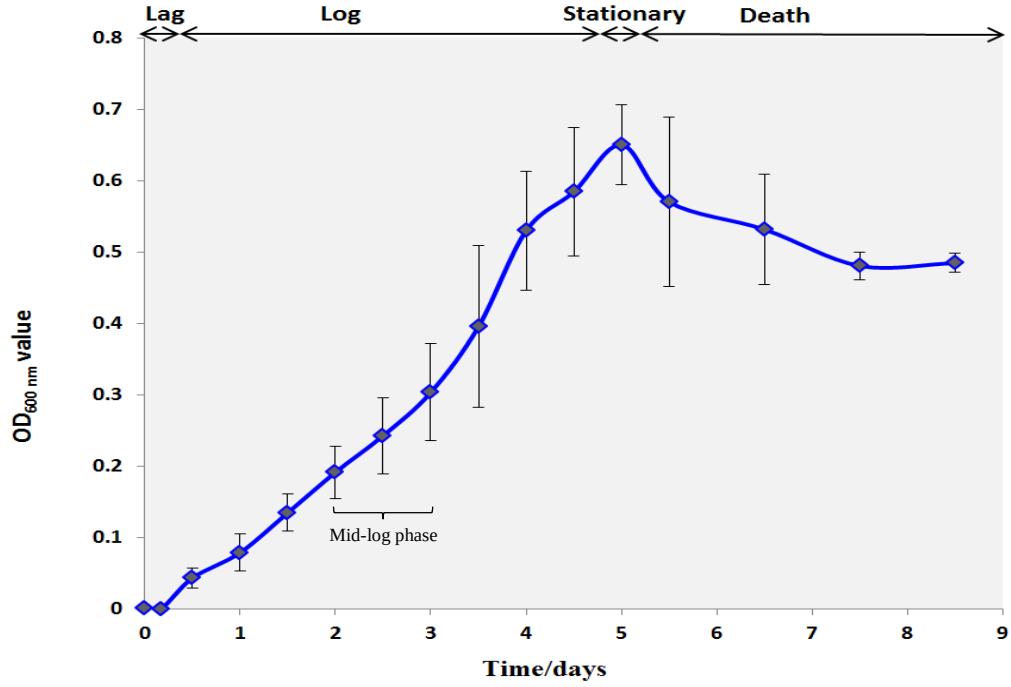


Figure 4.1: **Growth curve of *C. concisus* ATCC 51562 monitored by OD_{600}.** *C. concisus* ATCC 51562 was cultured in BHI and OD_{600} readings were taken at intervals for up to 9 days, data points represent the mean of three independent experiments; error bars show SEM. The curve presents mid-log phase at 2.5 days.

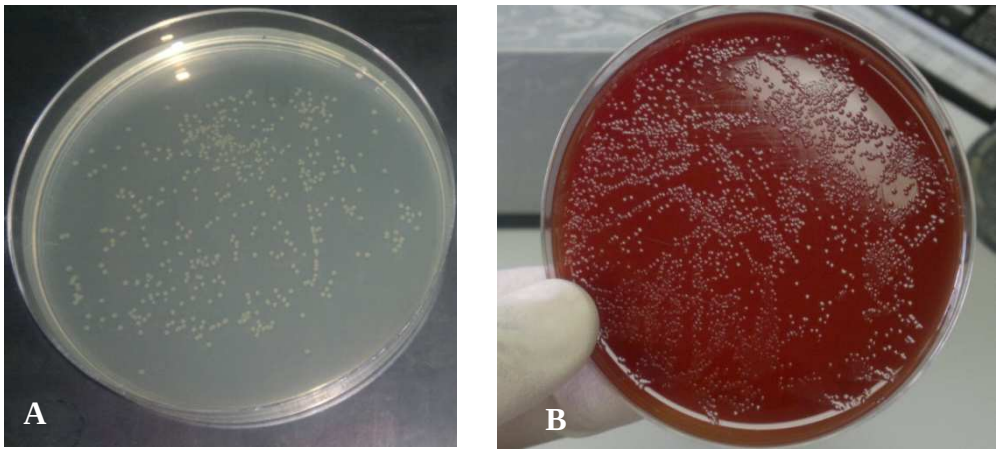


Figure 4.2: Growth of *C. concisus* on Columbia agar base (CA) without the addition of blood. Three day-old *C. concisus* colonies on a CA plate (A) and on a HBA plate containing 5% defibrinated horse blood (B). The colonies appear grey in colour and 1-2 mm in size on both plates.

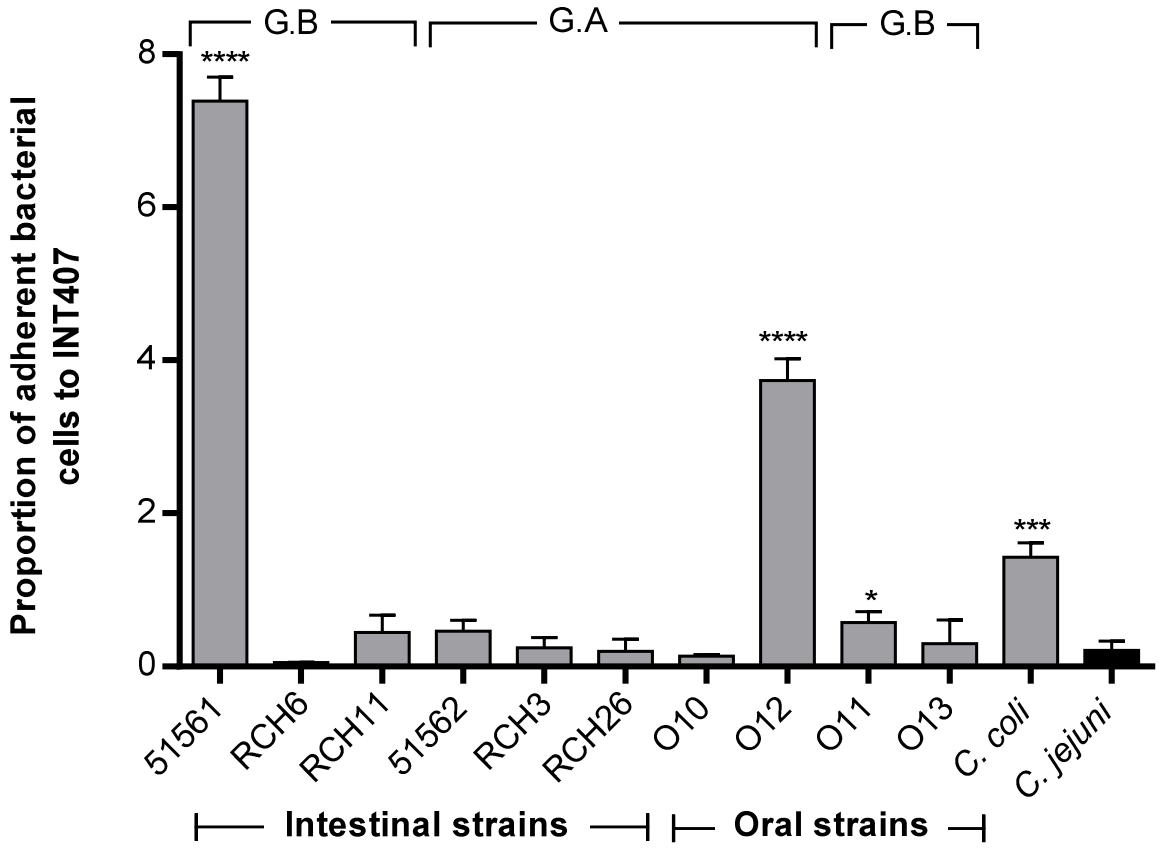


Figure 4.3: Adherence of *C. concisus* isolates to INT407 epithelial cells. Bacterial strains were mantained with INT407 cells for 6 h. Bars represent the mean of the percentage of adherent bacterial cells. The data are representative of three independent experiments performed in triplicate; error bars represent SEM. The adherence value of each *Campylobacter* spp. strain was compared to *C. jejuni* 81116 (the black bar) to calculate the significance. The number of stars represent the power of statistical significance (*: P <0.05, **: P <0.01, ***: P <0.001 and ****: P <0.0001). 51561 & 51562: *C. concisus* reference strains ATCC 51561 and 51562. RCH: *C. concisus* faecal isolates collected from the Royal Melbourne Children's Hospital. O12, O10, O11 and O13 were oral isolates. *C. coli* is *C. coli* NCTC 11366. G.A: genomospecies A. G.B: genomospecies B. All *C. concisus* tested isolates were able to adhere to INT407 cells with similar percentage to *C. jejuni* or higher.

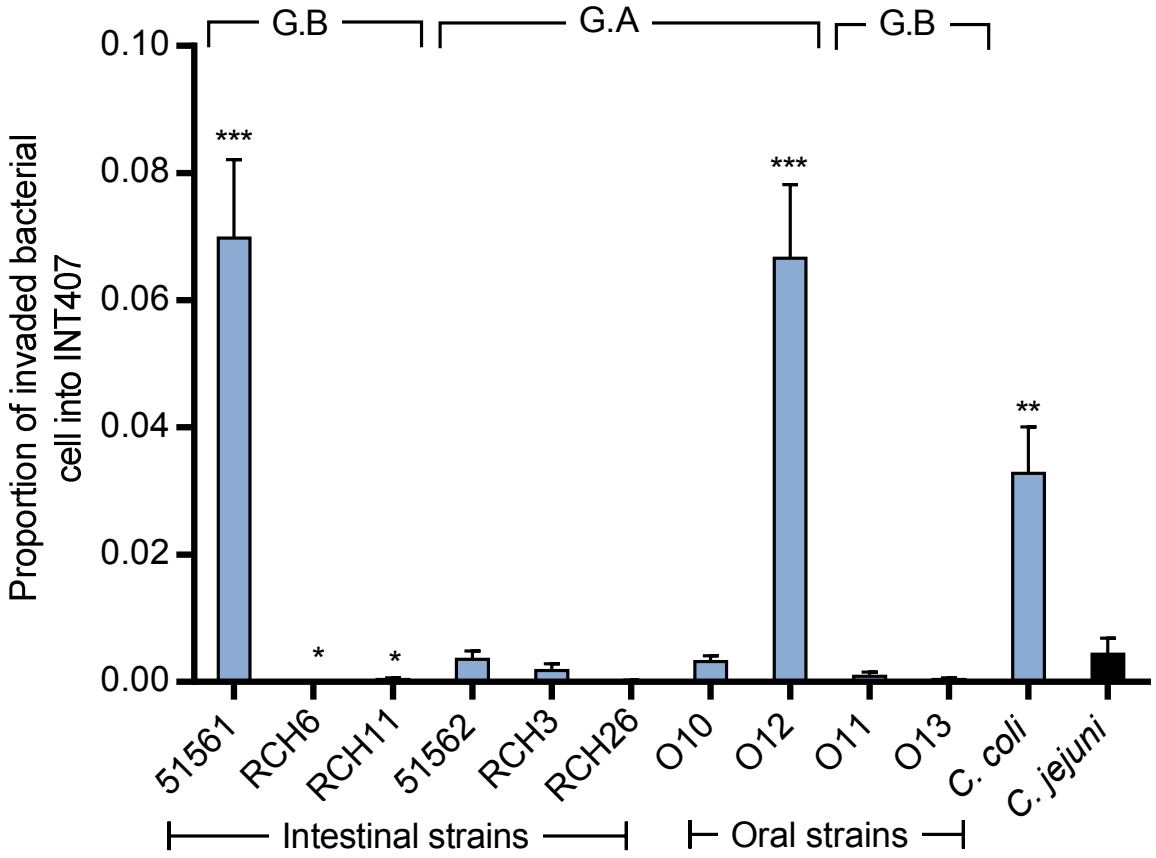


Figure 4.4: Invasion of *C. concisus* isolates into INT407 epithelial cells. Bacterial strains were mantained with INT407 cells for 6 h. Bars represent the mean of the percentage of invaded bacterial cells. The data are representative of three independent experiments performed in triplicate; error bars represent SEM. The invasion value of each *Campylobacter* spp. strain was compared to *C. jejuni* 81116 (the black bar) to calculate the significance. Stars represent the power of statistical significance (*: P <0.05, **: P <0.01 and ***: P <0.001). 51561 & 51562: *C. concisus* strains ATCC 51561 and 51562. RCH3, RCH6, RCH26, and RCH11: *C. concisus* faecal isolates collected from the Royal Melbourne Children's Hospital. O12, O10, O11 and O13 were oral isolates. *C. coli* is *C. coli* NCTC 11366. G.A: genomospecies A. G.B: genomospecies B. All tested isolates were able to invade INT407 cells with different proportions. The invasion proportion of RCH6 and RCH11 (possessing *zot*) was significantly lower than *C. jejuni* 81116.

The results showed that *C. jejuni* 81116, *C. concisus* isolates (O10, and O12) and *C. coli* NCTC 11366 were invasive strains. Moreover, there was no statistically significant difference in the invasive index values of two *C. concisus* isolates (O10 and O12) and *C. coli* NCTC 11366 as compared to *C. jejuni* 81116 (P >0.05), while other *C. concisus* isolates demonstrated a significantly lower invasive index values (P <0.05) as shown in Figure 4.5. Interestingly the lowest invasive index values were recorded for *C. concisus* clinical isolates (RCH6, RCH11 and RCH26**)** that possessed *zot*, while the oral isolates (O10 and O12) had the highest invasive index values.

Collectively, although *C. jejuni* 81116 and *C. concisus* O10 (an oral isolate) showed low incidences in adherence and invasion, their invasive index values were significantly higher, meaning that most of the attached cells were able to translocate into INT407 cells. The invasive index value of 0.95 for *C. concisus* ATCC 51561 was the highest compared to other *C. concisus* clinical isolates. Overall, two isolates (O10 and O12) from *C. concisus* genomospecies A had invasive (index invasive values >1), while none of genomospecies B isolates reached the invasive index value. The data also suggested that the presence of *zot* did not enhance the invasive capabilities of *C. concisus* isolates in INT407 cells. Furthermore, the majority of *C. concisus* tested isolates were more likely to be adherent than invasive when compared to the adherence and invasion of *C. jejuni* 81116 and *C. coli* NCTC 11366.

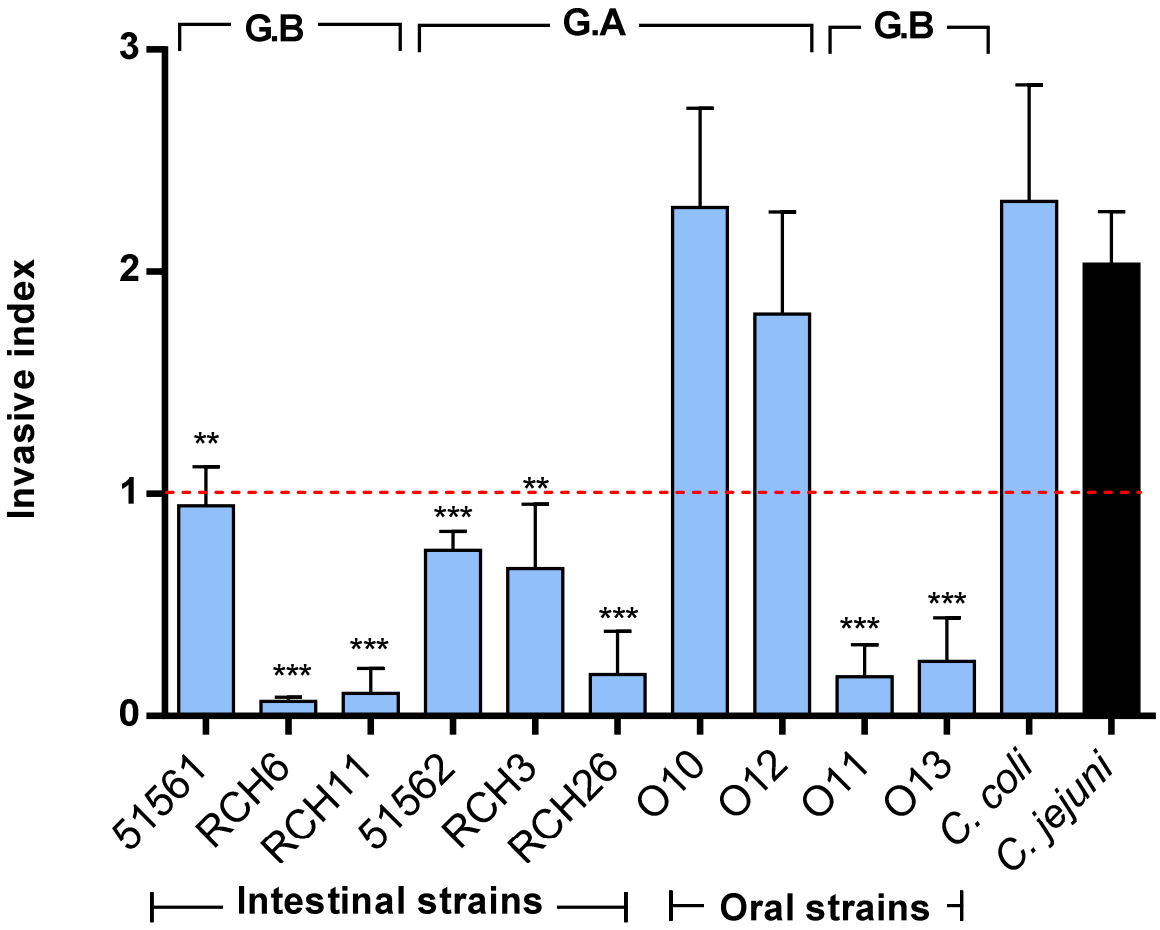


Figure 4.5: Invasive index value of *C. concisus* isolates infected into INT407 epithelial cells. Bars represent the mean of the invasive index values of bacterial cells. Data are representative of three independent experiments performed in triplicate; error bars represent SEM. The invasive index value of each *Campylobacter* spp. strain was compared to *C. jejuni* 81116 (the black bar) for significance calculation. Stars represent the power of statistical significant (*: P <0.05, **: P <0.01 and ***: P <0.001). 51561 & 51562: *C. concisus* reference strains ATCC 51561 and 51562. RCH3, RCH6, RCH26 and RCH11: *C. concisus* faecal isolates collected from the Royal Melbourne Children's Hospital. O12, O10, O11 and O13 were oral isolates. *C. coli*: *C. coli* NCTC 11366. G.A: genomospecies A. G.B: genomospecies B. Invasion index values (≥1) above the dashed horizontal line in red colour represent invasive *Campylobacter* strains, while invasion index values (<1) below the dashed horizontal line in red colour represent non-invasive *Campylobacter* strains. Invasive strains were *C. jejuni* 81116, two oral *C. concisus* isolates (O10 and O12) and *C. coli* NCTC 11366. All invasive *C. concisus* isolates were from genomospecies A and did not possess *zot*.

4.3.3 Expression analysis of putative virulence factors using semi-quantitative real time PCR (RT-PCR)

The lowest number of bacterial cells that yielded sufficient RNA for cDNA synthesis at a detectable level for the RT-PCR was $\geq 10^5$ CFU (Figure 4.6). The RT-PCR protocols were optimised to detect and amplify *cjaC, cjaA, dnaJ* and *zot* from cDNA. Purified RNA was also tested to ensure that residual genomic DNA was removed (Appendix XVIII). Melting curve analysis was used to confirm the specificity of SYBR green PCR products. As an additional control, PCR products obtained from the SYBR green assays were visualised by gel electrophoresis to ensure that a single product of the correct size was present. An example for the RT-PCR products is presented for the housekeeping gene (ATP synthesis F1 complex alpha subunit) in Appendix XVIII.

4.3.3.1 Expression analysis of putative virulence genes of *C. concisus* ATCC 51561 using semi-quantitative real time PCR

The gene expression level detected in *C. concisus* cultures grown on HBA was used as the baseline, and this value was arbitrarily set to 1.0. A minimum of 2-fold change in gene expression as compared to the expression on HBA was considered a significant change (either up or down regulated).

The expressions of *cjaC* and *cjaA* in *C. concisus* cells grown on CA were slightly up-regulated, but they were less than 2-fold when compared with baseline expression on HBA. This finding indicated that the expressions of these genes may not be significantly affected in the absence of blood in growth media. Moreover, the expressions of *cjaC* and *cjaA* were slightly down-regulated (<2-fold) in bacterial cells maintained in DMEM+INT407 cells and DMEM (Figure 4.7).

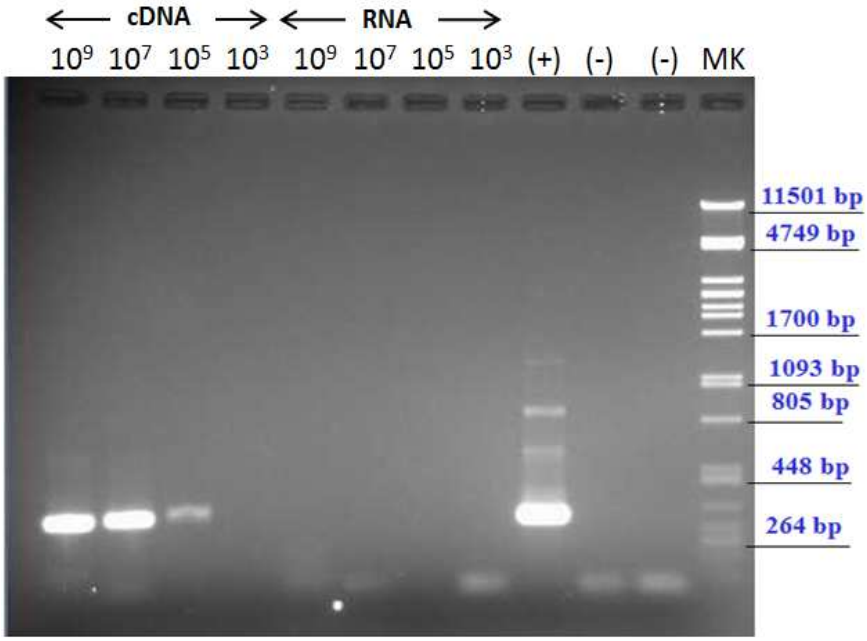


Figure 4.6: RT-PCR amplification products obtained from cDNA synthesised from RNA extracted from serially diluted bacterial suspensions. This experiment was performed to detect the lowest number of bacterial cells that yielded sufficient RNA for cDNA synthesis. RT-PCR amplification products of cDNA were obtained from *atpA* that was extracted from serial dilution of *C. concisus* ATCC 51562 cells. RT-PCR products collected from the bacterial dilutions were loaded on a 1.5% (w/v) agarose gel for electrophoresis. The gel was then stained in ethidium bromide and visualised by a UV trans-illuminator. PCR product size was 300 bp. Lanes 10^9, 10^7, 10^5 and 10^3: the bacterial dilutions. (+): control positive. (-): no template control. MK: lambda DNA marker. The lowest number of bacterial cells that yielded sufficient RNA for cDNA synthesis at detectable level for the RT-PCR was $\geq 10^5$ CFU.

Similar to *cjaC* and *cjaA*, there was no significant difference in the expression of *dnaJ* in bacterial cells grown on CA when compared to bacterial cells grown on HBA. However, the expression of *dnaJ* was significantly down-regulated in bacterial cells maintained in DMEM+INT407 cells and in DMEM (values of -3.5 and -6.7 respectively) (Figure 4.7). This may be due to the use of DMEM as a growth medium because HBA and CA may be more suitable for *C. concisus* growth than in DMEM.

Generally, the expression of all tested genes (*cjaC, cjaA* and *dnaJ*) in *C. concisus* cells maintained in DMEM+INT407 and in DMEM was significantly down-regulated compared to the expression in cells growing on CA (Figure 4.7). In addition, the expression of *dnaJ* in *C. concisus* cells maintained in DMEM was compared to the expression in cells maintained in DMEM+INT407 cells. It was found that there was a significant down-regulation in the expression of *dnaJ* in the bacterial cells maintained in DMEM compared to in the bacterial cells maintained in DMEM+INT407 cells (3.2-fold, $P < 0.05$) (Figure 4.7).

In summary, growth of *C. concisus* ATCC 51561 on CA showed no significant change in the regulation of the tested genes, which was supportive for growing *C. concisus* on CA. *DnaJ* encodes a stress protein and is considered as a virulence-associated gene. The results of expression studies for this gene showed that there was a significant down-regulation in *dnaJ* in the bacterial cells maintained in DMEM and in DMEM+INT407; however the down-regulation in DMEM alone was significantly lower than in DMEM+INT407, which suggested a possible role of *dnaJ* in *C. concisus* virulence.

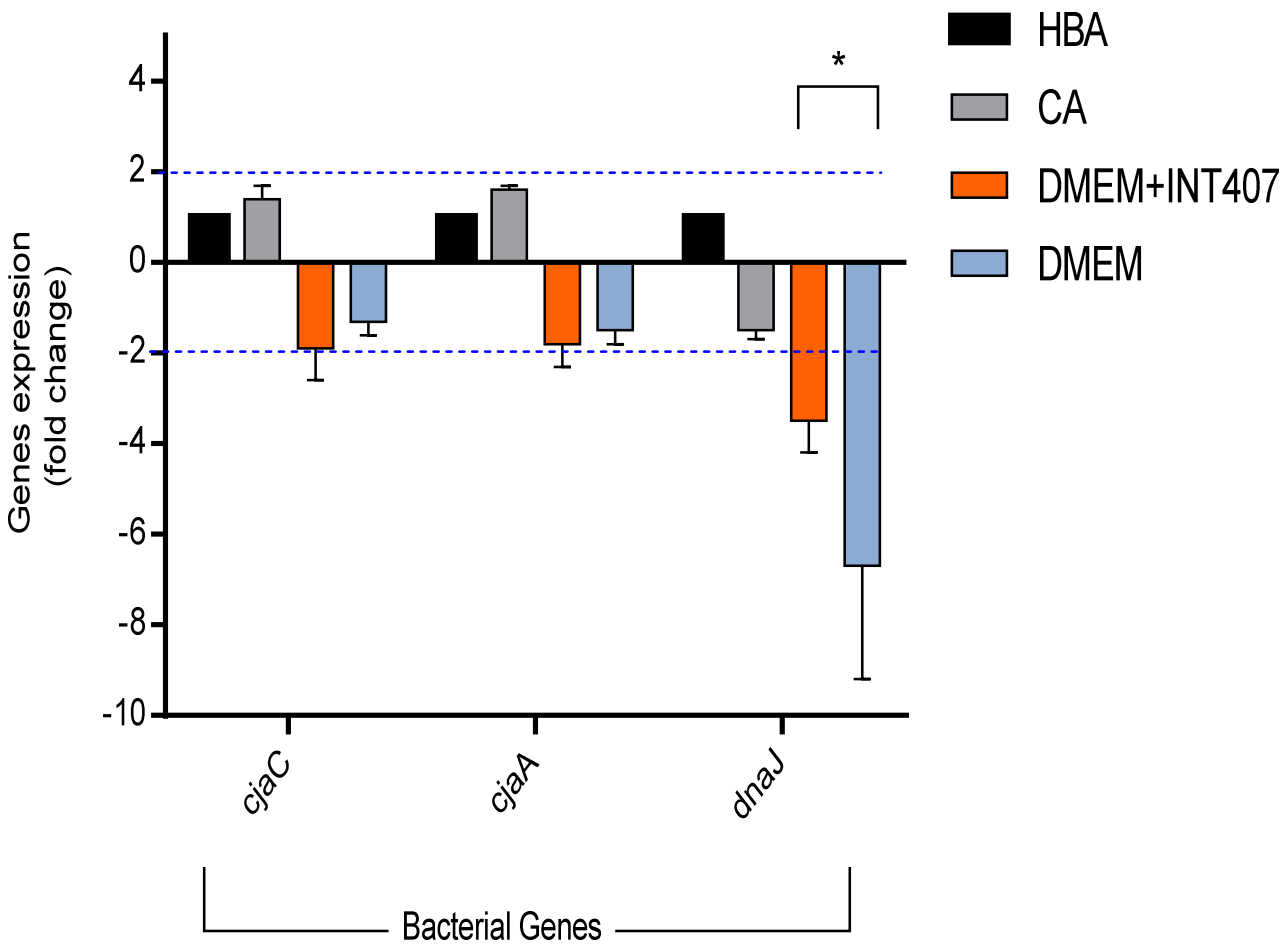


Figure 4.7: Gene expression of putative virulence genes of ATCC 51561 relative to expression on HBA. Bars represent the mean of the values of the fold change for *cjaC, cjaA* and *dnaJ* in bacterial cells grown on HBA, CA and, in bacterial cells mantained in DMEM+INT407 cells and DMEM medium alone. Data are representative of three independent experiments preformed in triplicate; error bars represent SEM. In this experiment relative gene expression from *C. concisus* ATCC 51561 grown on HBA was used as a reference (the black bar) for significance evaluation. The star represents the power of statistical significant (P <0.05). The two dashed blue lines show the two fold changes for up-regulation and down-regulation. CA: Columbia agar base. RNA was collected after 48 h in the bacterial growth on HBA and CA while it was collected after 6 h of bacterial maintained in DMEM+INT407 and DMEM. No significant change in the expression of *cjaC* and *cjaA* genes. The expression of *dnaJ* was significantly down-regulated in DMEM+INT407 and DMEM. Also, *dnaJ* was significantly down-regulated in bacterial cells maintained in DMEM compared to in bacterial cells maintained in DMEM+INT407.

4.3.3.2 Screening for the expression of the putative virulence genes in four *C. concisus* strains using semi-quantitative real time PCR.

After the expression level of putative virulence had been determined in the reference strain *C. concisus* ATCC 51561, ATCC 51562, RCH6 and RCH26 were selected for similar expression analysis. Gene expression of *C. concisus* ATCC 51562, RCH6 and RCH26 relative to that of ATCC 51561 was calculated. cDNA of *cjaA* could not be amplified from *C. concisus* ATCC 51562 and RCH26, as was also the case for genomic DNA (section 3.3.4). Other genes (*cjaC* and *dnaJ*) were found to be expressed in all tested strains but their gene expression levels varied (Figure 4.8). There were no significant differences in regulation of *cjaC* in any bacterial strains growing on CA compared to HBA.

C. concisus ATCC 51561 had the highest levels of gene expression amongst all tested strains when they were maintained in DMEM+INT407. *C. concisus* isolates RCH6 and RCH26 (possessing *zot*) showed a significant down-regulation in the expression of *cjaC*, *cjaA* and *dnaJ* on HBA, CA and DMEM+INT407 (exception *cjaA* of RCH26, which could not be amplified). The expression of *zot* could not be detected even in isolates known to possess the gene in chapter 3 (RCH6 and RCH26).

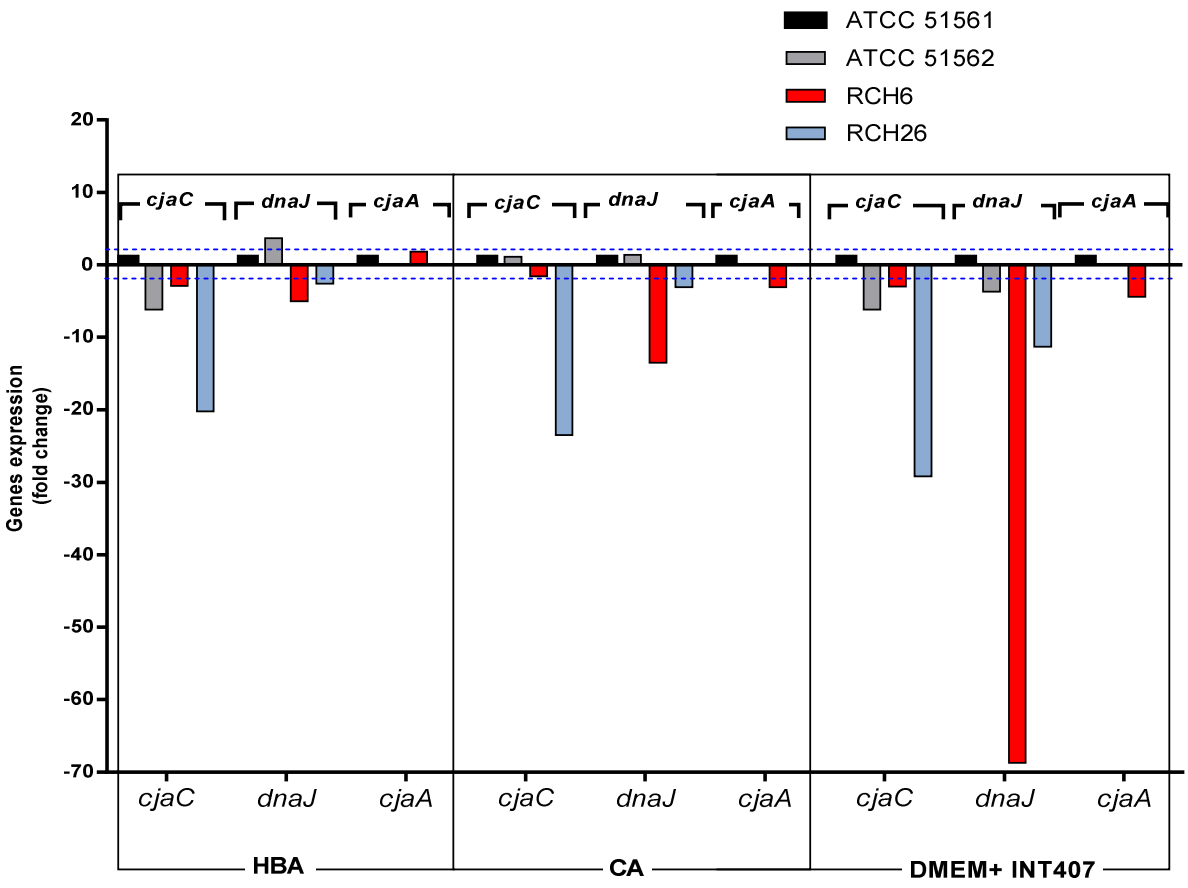


Figure 4.8: Gene expression of putative virulence genes in *C. concisus* relative to the expression of *C. concisus* ATCC 51561 grown in HBA, CA and with INT407 cells. The aim of the study was to investigate the expression of various genes (*cjaC*, *dnaJ* and *cjaA*) from *C. concisus* (ATCC 51562, RCH6 and RCH26) relative to *C. concisus* strain ATCC 51561. The bars are the fold changes of gene expression of one experiment that was performed and analysed once. The two dashed blue lines show the two-fold changes for up-regulation and down-regulation. The RNA was collected after 48 h growth on HBA and CA while it was collected after 6 h of bacterial cells maintained in to DMEM+INT407. *C. concisus* ATCC 51561 had the highest level of expression of all tested genes, but the difference was only statistically significant for bacterial cells maintained in DMEM+INT407. *C. concisus* RCH6 and RCH26 (possessing *zot*) had statistically significant down-regulation of their tested genes on HBA, CA and DMEM+INT407.

4.4 Discussion

Several studies have used different media to grow *C. concisus* including BHI broth and Brucella broth (Gunther *et al.* 2009; Lavrencic *et al.* 2012). In this study, *C. concisus* grew well on CA, a basic non-enriched medium without the addition of 5% defibrinated horse blood. Thus CA can be used for the maintenance of *C. concisus* strains in laboratories, particularly for resuscitating the bacterial isolates from frozen stocks, which can reduce the cost of *C. concisus* cultivation in laboratories. However, more studies are required to confirm whether CA can be used to retrieve *C. concisus* from clinical samples.

There had been no previous reports of growth curves for *C. concisus* in the literature, but growth curves for *C. jejuni* 81-176 in Mueller-Hinton (MH) broth have shown a rapid early growth peaking at 23 h (Sellars *et al.* 2002; Szymanski *et al.* 2002; Wright *et al.* 2009). In the present study, *C. concisus* growth in BHI was at a slower rate and required ~4 days to reach the peak of the growth curve (Figures 4.1). The mid-log phase in *C. concisus* was up to 2.5 days, longer than for *C. jejuni*.

In this study, *C. concisus* isolates demonstrated adhesion rates between 0.047% - 7.4% to INT407 cells. Previously, Kaakoush *et al.* (2011b) examined the adhesion and invasion capabilities of *C. concisus* isolates using different cell line (Caco-2 cells) at a MOI of 200 (three isolates obtained from intestinal biopsies of CD patients and four isolates obtained from faeces of patients with gastroenteritis), and reported adhesion rates between 0.11% - 4.6%. However, the faecal and oral *C. concisus* isolates used in this study showed lower invasion rates than reported by Kaakoush *et al.* (2011b). In using another cell line (CHO), Musmanno *et al.* (1998) found that two faecal *C. concisus* isolates were unable to adhere to and invade this cell line although one of the isolates was able to produce a cytotoxic effect when introduced into CHO cell line. The difference in the proportion of invasion between *C. concisus* isolates observed in these studies could be due to the reduced MOI of 100 (bacterial

cells per one host cell). It may also be the isolates that were obtained from intestinal biopsies were more invasive than faecal and oral isolates or using different cell lines.

In this study, the invasion and adhesion of *C. concisus* isolates did not show a clear difference between genomospecies A and B, or even between the oral or faecal isolates. Also, Kalischuk *et al.* (2011) did not demonstrate variations in the adherence to T84 cells between faecal genomospecies A and B isolates obtained from patients with diarrhoea and healthy controls. However, the same study indicated that the isolates belonging to genomospecies B were more invasive than genomospecies A. Moreover, the invasion rate for 46.6% (7/15) of *C. concisus* isolates was equivalent to that reported for *C. jejuni* 81-176 (Kalischuk *et al.* 2011).

In this study, two *C. concisus* oral isolates obtained from healthy volunteers were found to reach invasive index value >1, which has not been reported in the literatures. Ismail *et al.* (2012) found that 50% of *C. concisus* oral isolates obtained from IBD patients were invasive as they were able to reach the invasive index value, while none of *C. concisus* oral isolates obtained from healthy controls were invasive. The authors concluded that invasive *C. concisus* isolates colonised the oral cavity of patients with IBD only.

The INT407 cell line has been recognised to be contaminated with Hela cells (Lacroix 2008); however, it is still widely used in studying adhesion and invasion capabilities of campylobacters, in particular *C. jejuni* (Boehm *et al.* 2015; Chandrashekhar *et al.* 2015; Eucker *et al.* 2012; Javed *et al.* 2010). It was reported that *C. jejuni* was able to adhere to and invade Hela and INT407 cells with equivalent efficiency (Buelow *et al.* 2011; Eucker *et al.* 2012). Based on the invasion to Caco-2 and HT29-Cl.16E, Fearnley *et al.* (2008) found *C. jejuni* strains could be differentiated to hyper-invasive and low-invasive strains, relative differences also were retained when INT407 cells were used to test the same strains. In this current study, the results obtained from using INT407 cells were reproducible when three independent experiments were performed in triplicate. In addition *C. jejuni* 81116 and *C. coli*

NCTC 11366 were used as controls to compare the potential invasiveness of *C. concisus* isolates. However, conflicting results between studies may occur due to using different cell lines such as INT407 cells, Caco-2 and HT-29 for adhesion and invasion assays.

The putative virulence genes *cjaC* (histidine up-take), *cjaA* (cysteine up-take), *dnaJ* (virulence-association) were selected because they were predicted to be involved in bacterial survival in unusual environments or bacterial virulence. The results of the present study found that the expression of *cjaC* and *cjaA* was slightly changed in *C. concisus* ATCC 51561 cells grown on CA compared to the expression on HBA. Based on the expression of virulence factor, Malik-Kale *et al.* (2008) also found that the pathogenesis of *C. jejuni* can be induced by the change of growth conditions. The expression of *cjaC* was up-regulated, while the expression of *cjaA* was down-regulated in a *C. jejuni* strain grown on Mueller-Hinton agar containing 0.1% deoxycholate (Bile salt) (Malik-Kale *et al.* 2008). The current study found that there was no significant difference in expression of *cjaC* and *cjaA* when *C. concisus* ATCC 51561 was grown either on CA or when it was maintained in DMEM+INT407 or in DMEM alone, suggesting that expression of these genes is steady during *C. concisus* growth in different media and is not affected by introducing *C. concisus* to host cells.

Although, in the comparison of the expression results obtained from *C. concisus* ATCC 51561 cells grown on CA with the bacterial cells maintained in DMEM+INT407 or in DMEM showed small differences (within 2 to -2 fold, Figure 4.7), the results showed that the expression of *cjaC*, *cjaA* and *dnaJ* in bacterial cells maintained in DMEM+INT407 cells or in DMEM was significantly down-regulated compared with bacterial cells grown on CA. The variation in the results could be due to the time of RNA collection as in bacterial cells grown on CA was after 48 h, while the RNA of the bacterial cells maintained in DMEM+INT407 or in DMEM was collected after 6 h. Another point is that DMEM may not be a suitable medium for *C. concisus* growth as CA.

In this study, the expression of *dnaJ* in *C. concisus* ATCC 51561 cells maintained in DMEM was significantly reduced compared with the expression in those bacterial cells maintained in DMEM+INT407. Previously a *C. jejuni dnaJ* mutant was reported to be unable to colonise the chicken gut (Konkel *et al.* 1998). Moreover, *dnaJ* was reported to be up-regulated in *C. jejuni* cells grown in Mueller-Hinton broth incubated at 42°C as compared to 37°C (Stintzi 2003). Thus based on the results of this study and Konkel *et al.* (1998) study, the expression of *dnaJ* is possibly associated to the bacterial interaction with host cells.

In this study, the expression of the putative virulence genes was generally significantly higher in *C. concisus* ATCC 51561 than others isolates (ATCC 51562, RCH6 and RCH26) when the isolates were maintained in DMEM+INT407. Moreover, ATCC 51561 presented higher adhesion and invasion proportion values than ATCC 51562, RCH6 or RCH26. *C. concisus* ATCC 51561 was also the most invasive faecal isolate. Although *C. concisus* ATCC 51561 was isolated from the faeces of a healthy control, it was shown to be more virulent than other faecal isolates obtained from faeces of patients with diarrhoea. Furthermore, *C. concisus* isolates RCH6 and RCH26 (possessing *zot*) had a significant down-regulation in the expression of each of the three tested genes (*cjaC*, *cjaA* and *dnaJ*) on HBA, CA and DMEM+INT407. They were also amongst isolates that exhibiting the lowest values for adhesion and invasion proportion.

Wyszynska *et al.* (2004) demonstrated the presence of CjaC and CjaA proteins in the periplasm and suggested they could be exposed to the cell outer membrane of the bacterial cell. Although these proteins are exposed on the bacterial outer membrane, they do not seem to be involved in the interaction with host cells. In contrast *dnaJ* may be associated with bacterial virulence as the expression was significantly decreased in bacterial cells maintained in medium without the host cells, compared to those exposed to INT407 cells.

In summary, from the growth curve of *C. concisus* ATCC 51562 in Columbia broth, the mid-log phase occurred at 2.5 days. This information will assist future studies to determine suitable media or conditions for bacterial growth requirements and the phase of toxin production. In addition, *C. concisus* can be grown in ordinary synthetic medium such as CA without the addition of horse blood, which will reduce the cost of, and facilitate *C. concisus* growth in laboratories. Moreover, this study revealed that *C. concisus* of both genomospecies (A & B) were able to invade INT407 cells, but genomospecies A isolates displayed higher invasive index than genomospecies B isolates. The data obtained also showed that healthy volunteers can carry invasive *C. concisus* isolates in their oral cavity. Furthermore, these isolates had an invasive index value close to that of pathogenic *C. jejuni* 81116 and *C. coli* NCTC 11366 strains. Remarkably, *C. concisus* isolates possessing *zot* demonstrated the lowest invasive index value, suggesting that *zot* is not involved in host cell adherence and invasion, at least *in vitro*. Furthermore, to our knowledge it was the first relative expression assay study performed on the putative virulence genes such as *cjaC, cjaA* and *dnaJ*. It was found that the expression of these genes can be slightly induced by the alteration of the composition of the growth media. The *dnaJ* gene is probably a virulence factor as its expression was reduced when *C. concisus* introduced to a tissue culture medium without INT407 cells compared to the medium with INT407 cells. As *C. concisus* strains are well known to be divided into at least two main genomospecies, further studies on growth curves may include isolates from genomospecies B to investigate whether there is any difference in the growth curve patterns. This study provides a foundation for investigating relative gene expression by *C. concisus* and further studies should be undertaken in order to characterise additional predicted virulence genes.

Chapter 5 : Detection and characterisation of *C. concisus* in clinical samples collected from healthy adults and patients with IBD

5.1 Introduction

To date, only a few studies have investigated the presence of *C. concisus* in adults with IBD and there is a lack of information regarding *C. concisus* colonisation in sites throughout the entire human gastrointestinal tract in these patients. A recent study on adults with IBD attempted isolation of *C. concisus* from one site (caecal biopsies) and detection of *C. concisus* by PCR in four intestinal anatomic sites (Mahendran *et al.* 2011). Other studies were conducted on biopsies of paediatric patients with IBD collected from the distal colon (rectum/sigmoid) and caecum (Hansen *et al.* 2013; Zhang *et al.* 2009).

Certain groups of *C. concisus* could have possible pathogenic roles in IBD. The prevalence of genomospecies of *C. concisus* that colonise different sites of the human digestive tract has not been investigated in IBD patients (Hansen *et al.* 2013; Mahendran *et al.* 2011; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009). Additionally, there is controversy regarding which *C. concisus* strains or group is more virulent (Kalischuk *et al.* 2011).

Previous studies have reported very low isolation rate of *C. concisus* from intestinal biopsies (Mahendran *et al.* 2011; Zhang *et al.* 2009), which could be either due to low sensitivity of the culture techniques or that only low number of *C. concisus* colonise the gastrointestinal tract. Molecular techniques to amplify 16S rDNA have previously resulted in high detection rate (Mahendran *et al.* 2011; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009).

The purposes of the experiments described in this chapter were to:

I. Determine the prevalence of *C. concisus* in adults with IBD.

II. Determine the regions of the gastrointestinal tract colonised by *C. concisus*.

III. Determine whether there are any genomospecies differences between *C. concisus* strains colonising gastrointestinal and oral sites in IBD patients compared to control participants.

IV. Investigate the prevalence of *zot* (a putative virulence gene) in IBD patients and control participants.

V. Investigate the prevalence of *C. concisus* groups obtained from the detection of the DNA region between *cjaC* and the adjacent gene (CCC13826_0962) encoding a hypothetical protein in genomospecies B isolates to determine whether there was any association between those groups and IBD.

VI. Investigate the relationship between *C. concisus* isolates from the mouth and gastrointestinal tract of the same patient using SDS-PAGE.

5.2 Materials and Methods

5.2.1 Assessment of the effects of PicoPrep on *C. concisus*

PicoPrep (Fresenius Kabi, Australia) is sodium picosulfate powder that is normally used as an oral solution for bowel preparation prior to the endoscopy procedure. This experiment was conducted to find whether PicoPrep might inhibit and reduce *C. concisus* survival in intestinal specimens. *C. concisus* were grown on HBA using the disc diffusion method. *C. concisus* isolates (ATCC 51562, RCH6 and RCH24) were nominated as representatives of both *C. concisus* genomospecies (A and B), the experiment was performed in triplicate. A sachet content (15.5 g) of PicoPrep was dissolved as recommended for the bowel preparation in 250 ml of sterile dH_2O. Then, several dilutions were made as follows: 1:0, 1:2, 1:4, 1:8 and

1:10. Bacterial growth was initiated by freshly suspending 1-2 colonies into a tube containing 1 ml brucella broth and incubating under optimal conditions of *C. concisus* as stated in section 2.4.2 for 5 h. A HBA plate was inoculated by spreading 100 µl from the bacterial culture; following that, five sterile discs were placed on and were then saturated with 50 µl of one of the following concentrations of PicoPrep: 124 (1:0), 62 (1:2), 31.2 (1:4), 15.2 (1:8) and 5.2 (1:10) mg/ml. Plates were incubated under *C. concisus* growth conditions as described in section 2.4.2 for 4 days.

5.2.2 Recovery and survival of *C. concisus* after storage at 4°C

This experiment was performed to investigate the effect of low temperature (4°C) on the recovery and survival rates of *C. concisus* as this temperature can be used as a model for the transportation temperature or short storage between collection of biopsies and laboratory culture. *C. concisus* ATCC 51561 (a genomospecies B strain) and *C. concisus* ATCC 51562 (a genomospecies A strain) were nominated to reflect both genomospecies for this experiment.

Bacterial suspensions were prepared in 5 ml of Columbia broth (CB) from *C. concisus* strains grown for 48 h old HBA. The suspensions were adjusted to 0.1 OD_{600} and stored refrigerated at 4°C. To detect the survival rate at this temperature, *C. concisus* CFU value was determined after storage in CB at 4°C for up to 15 days; and samples were tested at times 0 h, 7 h, 24 h, 48 h, 120 h, 192 h, 288 h and 360 h.

5.2.3 Sample size

In order to determine the sample size that is necessary to achieve statistical significance, power analysis was conducted with assistance of the RMIT University Statistics Consultancy Group. The power analysis was conducted using GPower 3.1 (Faul *et al.* 2007), this package

very comprehensive for estimation of sample size. Individual power analyses were conducted for each of the four specimens (biopsy, faeces, gum swab and blood) that were to be collected from each patient at the same time. The sample size estimate was based on the results achieved in previous studies found in the literature (Man *et al.* 2010b; Zhang *et al.* 2009) where the bacterium was identified in intestinal biopsies and stool samples from patients and control groups which were nominated at the start of the study. Power analysis indicated that 117 samples were required for the patient (study) group, and 83 samples from control group.

5.2.4 Recruitment of participants

Participants in this study were recruited from patients attending the Gastroenterology clinic at Austin Health, Heidelberg, Victoria. Laboratory work was undertaken at RMIT University, Melbourne, Victoria. The lead clinician was Dr. Georgina Paizis. Ethics approval for this study was granted by Austin Health HREC (No. H2012/04518). The approved application was then endorsed by RMIT University HREC.

5.2.5 Criteria for inclusion in the study

Participants considered to participate in the study were adults deemed to require endoscopy procedures for clinical indications (such as for the diagnosis of intestinal diseases) purposes. The participants were divided into two groups as follows: IBD patients who were suffering from either CD or UC, and control participants (those who were undergoing endoscopic surveillance for family history of colorectal cancer or polyps, or a personal history of colonic polyp).

5.2.6 Sample collection

Participants were given relevant written information on this study, purpose, risks and potential benefits, and signed a consent form prior to sample collection. This study involved collection of intestinal biopsies, stool, blood and gum samples from each participant at the same session as the endoscopy procedure.

Standard sized of intestinal biopsies (3-5 mm) were collected from different sites of the gastrointestinal tract during the endoscopy procedure. In most of the participants, samples were collected from seven intestinal sites: terminal ileum, caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum (Figure 5.1). For a few participants, two other sites (duodenum and stomach) were also included as shown in Figure 5.1. The biopsies were taken by endoscopy using standard procedures; clean washed forceps was used between each biopsy of each participant (Figure 5.1). The collected biopsy was then kept in a sterile container containing CB in ice.

Faecal samples were aspirated by the endoscopy instrument during the procedure and collected into sterile containers. Gum samples were collected during the endoscopy session using the Copan E-swabs (Copan Italia, Italy). Blood samples were collected through a cannula inserted during the procedure as part of colonoscopy preparations. The blood sample was collected into a sterile tube without anticoagulants.

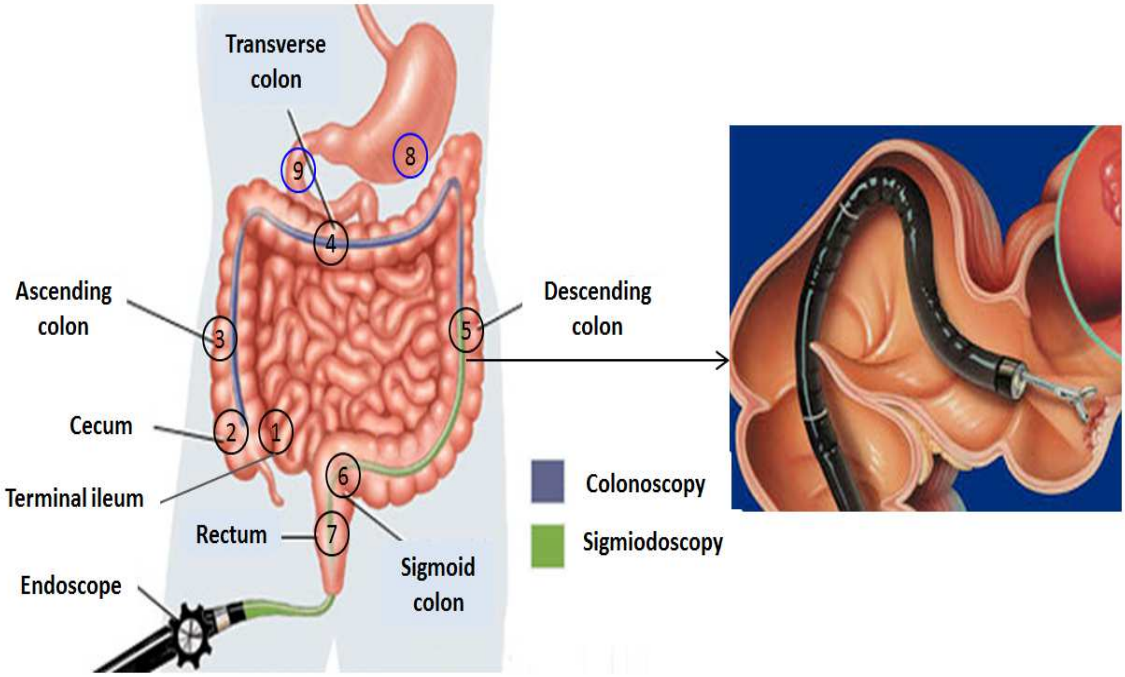


Figure 5.1: Anatomic sites of the intestinal biopsies collected from participants enrolled in the study. Biopsies were collected by endoscopy. The right photo shows the standard forceps for biopsy collection (Wogahn 2015). Sites 1-7 are for the intestinal biopsies collected routinely by endoscopy from most of the participants while sites 8-9 were stomach and duodenum biopsies, which were only collected from four participants by gastroscopy (VanAmburg 2014).

5.2.7 Sample coding

The participants' information was collected at Austin Health and was specifically labelled to be re-identifiable only by Austin Health's staff. Austin Hospital codes and patient names were not provided in this study. Instead, codes were used to refer to Austin Hospital (AUS) such as, stool (S), biopsy (B), gum samples (G) and blood (BO). The bacterial isolates were designated with lower case letters as a, b, c. For example, specimens from participant 1 and isolates were AUS1-Sa, AUS1-Ba and AUS1-Ga, respectively.

5.2.8 Processing of clinical samples in the laboratory

All procedures on collected samples were performed in a class II biological safety cabinet.

I. Intestinal biopsies

In general, each fresh biopsy (immediately after the collection) was cut by a new sterile scalpel into several parts (3-4 parts) as following:

1. Part 1: was used for DNA extraction to detect *C. concisus* DNA.
2. Part 2: was cultured directly onto HBA medium by streaking to isolate viable *C. concisus* (Figure 5.2).
3. Part 3: was inoculated into enrichment broth (3 ml Ham's F-12 with 10 µl/ml vancomycin) to isolate *C. concisus* as described by Kaakoush *et al.* (2011b) (Figure 5.2).
4. Part 4: any extra material left from the biopsy was stored in -80°C.

Parts 2 and 3 cultures were incubated for 48 h under *C. concisus* growth conditions as stated in section 2.4.2. After the incubation of part 3 cultures, 200 µl of the broth was introduced onto a cellulose acetate filter (0.65 µm) placed on HBA with 10 µl/ml vancomycin. The filter was kept for 45-60 min to allow *C. concisus* to pass through then it was carefully removed and the plates were streaked and incubated as described in section 2.4.2.

II. Gum samples

In this study, 'the Cape Town protocol' described by Lastovica *et al.* (2000) for *C. concisus* isolation from faecal samples was modified and used for *C. concisus* isolation from gum samples as follows: approximately a 100 µl of homogenised liquid medium from the Copan E-swab was added onto a cellulose acetate filter (0.65 µm) placed onto HBA (three HBA plates were prepared for each swab using a total of 300 µl of the liquid medium). The filters were removed and discarded after 45-60 min (Figure 5.2). The inoculated plates were incubated as explained in section 2.4.2. The remaining 400 µl of the E-swab liquid medium was used for DNA extraction to detect *C. concisus* DNA.

III. Faecal samples

Faecal samples were treated based on their consistency to retrieve *C. concisus* by culture. Semi-solid samples were liquefied in Columbia broth and were diluted at ~1:3; whereas, liquid samples were not diluted. Each sample was cultured onto three HBA plates by adding ~150 µl onto cellulose acetate filter (0.65 µm) placed onto a HBA plate for 45-60 min to allow the bacterium to swim through the filter to the HBA (Figure 5.2) as described in 'Cape Town protocol' (Lastovica *et al.* 2000). The plates were incubated as described previously in section 2.4.2 for growing *C. concisus*. One hundred and fifty milligram from the remaining faecal sample was used for DNA extraction.

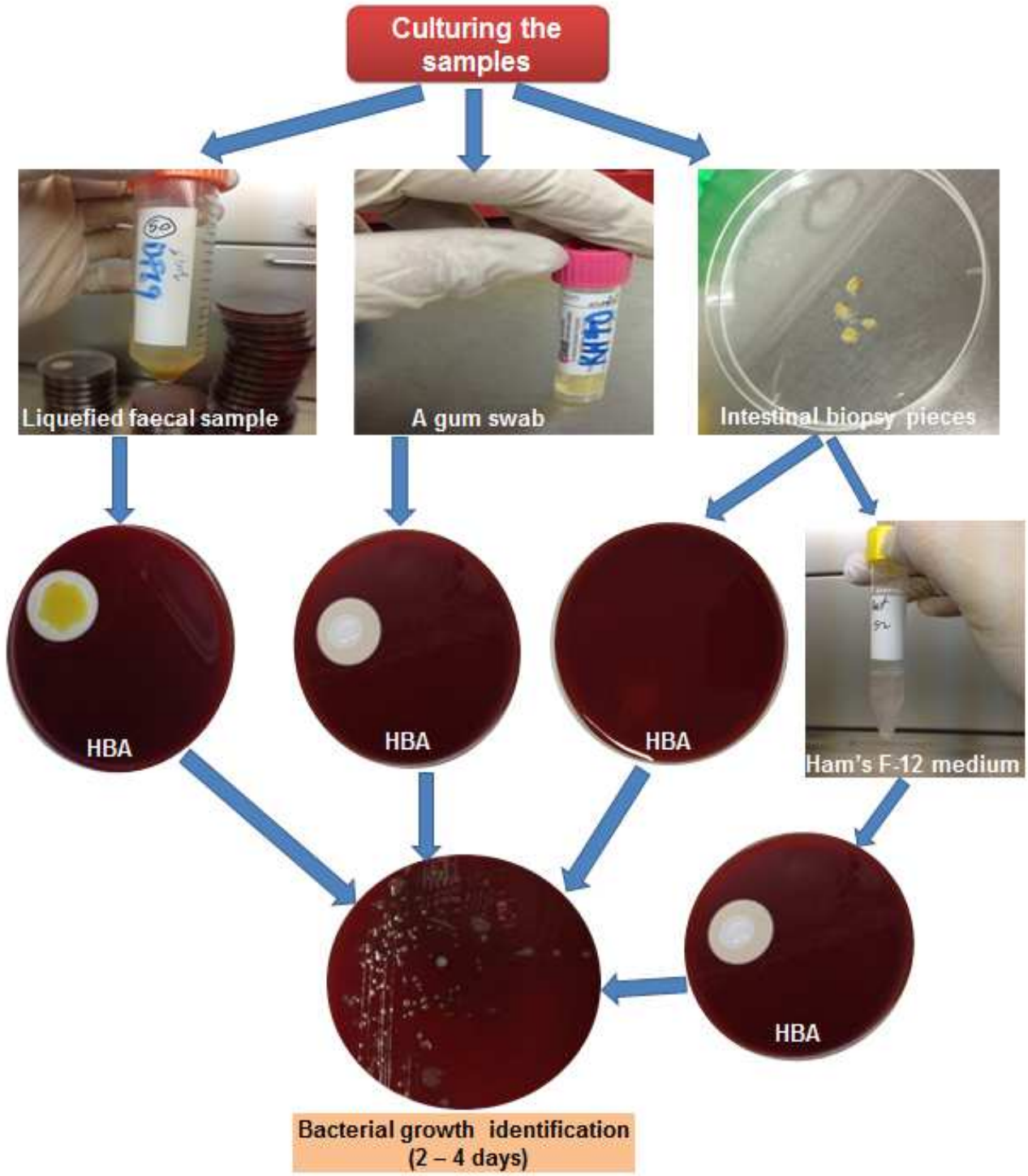


Figure 5.2: Processing strategy of collected biopsies, faecal and gum samples for isolation of *C. concisus*. Faecal specimens were cultured following the 'Cape Town protocol' by filtration onto HBA (horse blood agar). The 'Cape Town protocol' was modified and used to isolate *C. concisus* from gum samples. Intestinal biopsies were cut to 4 pieces. The first and second pieces were cultured directly onto HBA and into Ham's F-12 medium. The third piece was used for DNA extraction. The fourth piece was stored at -80. Then after 2 days incubation, Ham's F-12 medium contained bacterial growth was filtrated onto HBA.

I. Blood

Blood samples were allowed to clot and then centrifuged at 2000 g for 10 min to collect the serum, which was stored at -80°C. The serum was not included in this study because of time limitations.

All cultured HBA plates (from biopsies, faeces and gum samples) were incubated for 2-4 days; then examined to detect any suspected *C. concisus* colonies. Each suspected pure colony was subcultured on a HBA plate to propagate the bacterial isolates using the identification tests stated in section 2.4.3 and for DNA extraction for PCR confirmation.

The identity of the obtained *C. concisus* isolates was confirmed by Multiplex PCR (m-PCR) as previously described by Huq *et al.* (2014). In the m-PCR, four primer sets including (C412-F/C1288-R, Pcisus (5 & 6), HipO-F/HipO-F and CC18F/CC519R) were used to detect *Campylobacter* genus, *C. concisus, C. jejuni* and *C. coli*, respectively (Table 2.2). The m-PCR protocol was performed as described in section 2.7.

DNA extraction from intestinal biopsies and gum samples was performed using the 'Isolate II Genomic DNA kit' (Bioline, Australia), while DNA extraction from faecal samples was done using the 'Isolate Faecal DNA Kit' (Bioline, Australia) following the manufacturer's instructions.

5.2.9 Direct detection of *C. concisus* in the clinical samples by PCR

5.2.9.1 DNA estimation

The DNA quantity of each sample was standardised by diluting the DNA in nuclease free water (Bioline) prior to performing PCR. In each 25 µl PCR reaction, 40 ng DNA was used.

5.2.9.2 PCR conditions

C. concisus DNA was detected using nested PCR (two step) that was described by Man *et al.* (2010b). The two primer sets were the *Campylobacter* genus-specific primer set (C412F and C1228 R for PCR step I) and *C. concisus* species-specific primer set (Concisus F and Concisus R for PCR step II) (Table 2.2). The amplification protocol was achieved by two steps as follows:

PCR Step I

This step was performed as described by the method of Linton *et al.* (1996) which amplified an ~800 bp fragment of the 16S rDNA gene using the primer set C412F and C1228.

PCR Step II

This step was performed as described by Man *et al.* (2010b) by amplification of a 560 bp fragment inside the 16S rDNA gene using the Concisus-F and Concisus-R primer set.

The sequences of the primer sets C412F/C1228R and Concisus-F/Concisus-R are described in Table 2.2. PCR I and II conditions as they were described by Man *et al.* (2010b).

5.2.10 Genomic typing of *C. concisus* isolates obtained from clinical samples

Determination of *C. concisus* genomospecies A or B was as described by Istivan *et al.* (2004) and detailed in section 3.2.2.

5.2.11 Detection of genomospecies B directly in the collected clinical samples

A new nested PCR technique (GB-PCR) was developed to detect genomospecies B directly from clinical samples. GB-PCR was designed to amplify the *cjaC* region. The first PCR step (GB-PCR1) was to amplify 800 bp region of *cjaC* using primer set I; then the second step PCR (GB-PCR2) was to amplify 300 bp (inside the 800 bp region of *cjaC*) using primer set II. The sequences and the locations of these primer sets (I and II) were described in Table 2.2.

GB-PCR1 reactions were performed in a volume of 25 µl using 5 pmol of each primer (GeneWorks, Australia), using the 'MyTaq^{TM}DNA' Polymerase (Bioline, Australia). The reaction mix contained 1 µM of deoxy-nucleotidetriphosphates mixture, 3 mM MgCl2, 0.7 U of Taq polymerase, and 40 ng of the extracted DNA. Thermal cycling conditions were as follows: 2 minutes at 95ºC, thirty cycles of 15 seconds at 94°C, 15 seconds at 54°C and 30 seconds at 72°C, followed by the final extension of 7 min at 72°C.

GB-PCR2 reactions were performed as above except for the DNA template concentration, which was 1 µl from PCR1 for biopsies and faecal samples, while in gum samples 1 µl was used after a dilution of 1:100 (as *C. concisus* DNA concentration is very high in gum samples compared to other samples). Thermal cycling conditions were as follows: 2 minutes at 95°C, forty cycles of 15 seconds at 94°C, 15 seconds at 64.9°C and 30 seconds at 72°C, followed by the final extension of 7 min at 72°C.

5.2.12 Detection of *zot* in the *C. concisus* isolates obtained from clinical samples

The PCR technique was performed as described in section 3.2.5 using ZoT primer set (Kalischuk *et al.* 2011) to amplify (355 bp) *zot*. Then for *zot* sequencing, genomic DNA was subjected to another PCR using primer set ZoT-F/ZotA2-R to amplify 1055 bp of the gene sequence as described previously by Mahendran *et al.* (2013) and stated in Table 2.2. PCR conditions were described in sections 2.7. The PCR products were purified and prepared for DNA sequencing as described in section 2.8.

5.2.13 Detection of *zot* directly in clinical samples by nested PCR

A new nested PCR (Z-PCR) technique was developed to detect *zot* directly from clinical samples in two steps. Two primer sets were designed to amplify *zot*. In the first PCR step (Z-PCR1) the primers were ZoT-F and ZotA2-R (Table 2.2), which was previously designed to

amplify a 1055 bp amplicon (Kalischuk *et al.* 2011; Mahendran *et al.* 2013). In the second PCR step (Z-PCR2) primer set (ZotB) was designed in this study to amplify 300 bp of *zot* (Table 2.2).

The Z-PCR1 reaction was performed in a volume of 25 µl using 10 pmol of each primer (GeneWorks, Australia), using the 'MyTaq^{TM}DNA' Polymerase (Bioline, Australia). The reaction mix contained 1 µM of deoxy-nucleotidetriphosphates mixture, 3 mM $MgCl_2$, 0.7 U of Taq polymerase, and 40 ng of DNA extracted from each clinical sample. Thermal cycling conditions were as follows: 2 minutes at 95°C, thirty cycles of 30 seconds at 94°C, 30 seconds at 52°C and 2 min at 72°C, followed by the final extension of 7 min at 72°C.

The Z-PCR2 reaction was performed as stated in PCR2 in section 5.2.11 except for the DNA template, which was 1µl from Z-PCR1. Thermal cycling conditions were as follows: 2 minutes at 95°C, forty cycles of 30 seconds at 94°C, 30 seconds at 58°C and 1 min at 72°C, followed by the final extension of 7 min at 72°C.

5.2.14 Characterisation of the *cjaC* flanking region in *C. concisus* isolates

Detection of the DNA region between *cjaC* and its adjacent gene (CCC13826_0962) which encodes for a hypothetical protein was performed using primer set VI as described in section 3.2.4. This was undertaken because some *C. concisus* isolates from genomospecies B were found to have different nucleotide sequences as indicated in chapter 3 and were allocated into 3 distinctive groups. PCR conditions and protocol were also described in section 2.7.

5.2.15 SDS-PAGE to investigate the similarity or differences between *C. concisus* oral and enteric isolates obtained from the same participant

Whole cell lysates for SDS-PAGE were prepared to investigate the relatedness between *C. concisus* isolates obtained from gum, faeces and intestinal biopsy samples collected from the

same participant at the same session. The SDS-PAGE technique was undertaken as described in section 2.13.

SDS-PAGE was used to compare the protein profiles obtained from multiple isolates from different sites of the same patient. Although diversity of DNA sequence among different isolates was demonstrated, the differences may not be translated into differences in protein profiles, as some could be silent mutations. *C. concisus* isolates were also found to be genetically heterogeneous as shown in chapter 3. The use of SDS-PAGE profiles might therefore show less dissimilarity between the isolates, which may give a better view on the relatedness between isolates colonising different sites of the same patient at the same time.

5.2.16 Data analysis

Statistical analysis was performed with GraphPad Prism 6 software (USA) using Chi-square (2-tailed test) or the Fisher's exact test (2-tailed test) to calculate *P* values between IBD patients and control participants. Two-way ANOVA was used for the comparison of the survival rate in between the strains of *C. concisus*. A value of $P < 0.05$ was considered significant. The NCBI data-base site was used to align and find the tested DNA regions and amino acid sequences. The Clone manager suite software was used for the alignment of sequences. MEGA software was used for the tested gene sequence alignment and for generating phylogenetic trees by the neighbour-joining dendrogram (Felsenstein 1989).

5.3 Results

5.3.1 Assessment of the effects of the PicoPrep on *C. concisus* growth

No zone of inhibition was observed around the discs with different concentrations of the PicoPrep. Therefore, it was concluded that the PicoPrep had neither bacteriostatic nor bactericidal properties against *C. concisus* isolates *in vitro* at ≤124 mg/ml and would not be expected to interfere with isolation of *C. concisus* from the intestinal samples in this study.

5.3.2 Survival of *C. concisus* in the specimen transport conditions at 4°C

The recovery rate of *C. concisus* ATCC 51562 was $\log_{10}$ 8.05 and $\log_{10}$ 7.83 for *C. concisus* ATCC 51561 at the start (0 h) (P >0.05). Furthermore, the bacterial number recovered for both strains was not affected significantly by 7 h storage at 4°C compared to the starting inoculum (P >0.05). However, the number was reduced significantly after 7 h for *C. concisus* ATCC 51562 and after 24 h for *C. concisus* ATCC 51561 compared to the starting inoculum (P <0.05) (Figure 5.3). Thus, it was concluded that the number of *C. concisus* recovered was not significantly reduced during the first 7 h storage at 4°C. In summary, the recovery of *C. concisus* from clinical samples was not significantly affected for ≤7 h for either genomospecies A or B, enabling reasonable time for clinical sample collection, transport on ice and processing.

The comparison in the survival rate between the two strains over 360 h storage at 4°C revealed that there was no statistically significant difference in the recovery rate (P >0.05) during the first 48 h storage. However, the survival rate of *C. concisus* ATCC 51561 was reduced significantly compared to *C. concisus* ATCC 51562 between 120 h-360 h (P <0.05) (Figure 5.3). The survival of genomospecies A isolates is possibly longer than genomospecies B isolates.

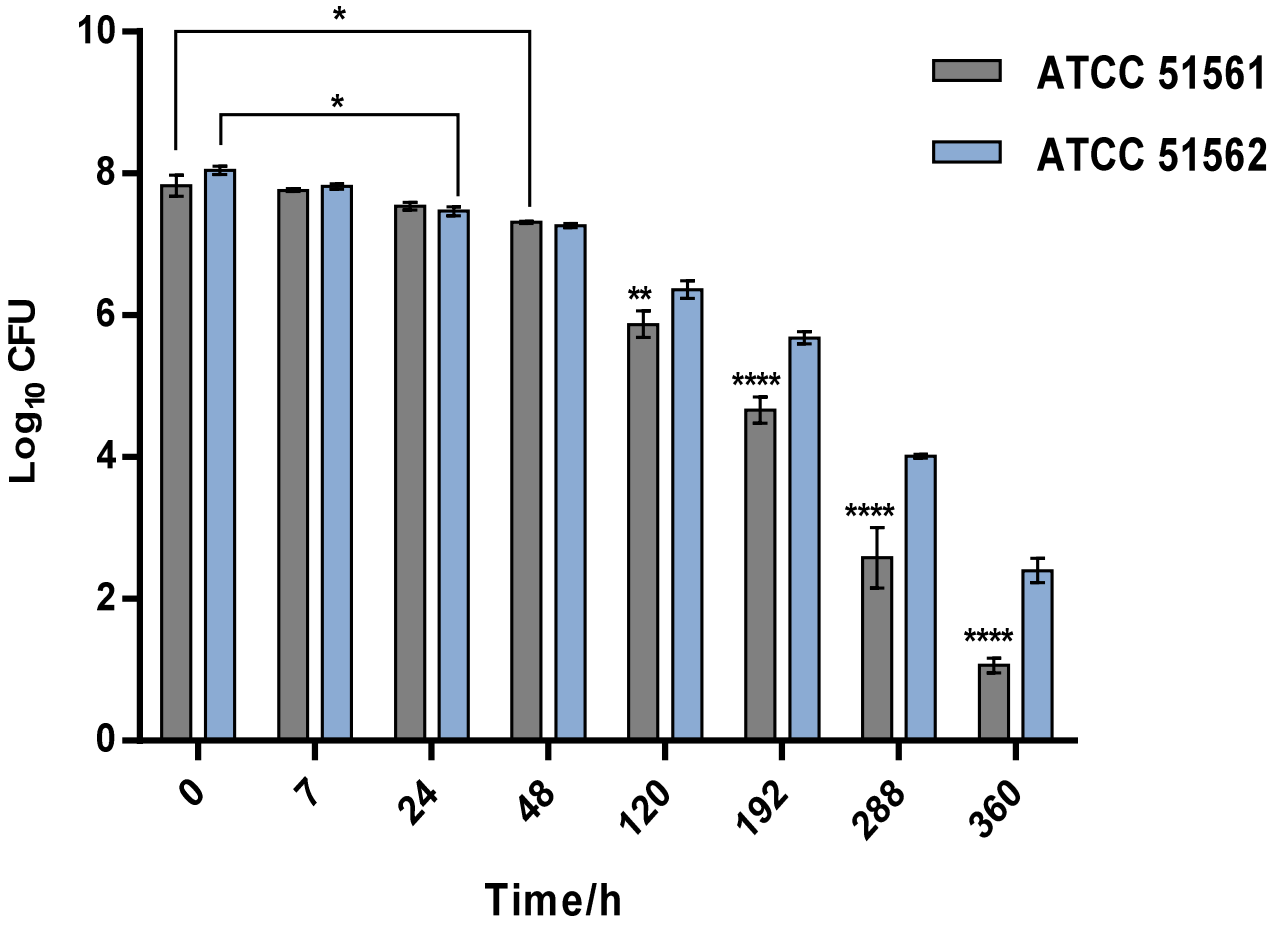


Figure 5.3: Retrieval and survival of *C. concisus* at low/transport temperature (4°C). Recovery rate of *C. concisus* strains ATCC 51561 and 51562 suspended in CB and storage at 4°C for 360 h. The survival of the strains at 4°C was monitored by $\log_{10}$ of CFU. The values represented by the bars are the mean of the CFU values obtained from three independent experiments. Error bars represent SEM. The number of stars represent the power of statistical significance (*: P <0.05, **: P <0.01, ***: P <0.001 and ****: P <0.0001). There was no significant difference in the reduction of survival number during the first 7 h for both *C. concisus* strains (ATCC 51561 and 51562). There was a significant difference between the two strains after 120 h (P <0.05).

5.3.3 Patient demographics

Fifty one participants were enrolled in this study, including 37 IBD patients (26 CD and 11 UC) and 14 control participants who attended the Gastroenterology clinic at Austin Health between August 2012 and February 2014. The average age of the IBD patients and control participants was 46.5±18 and 58±18 years, respectively. The average age of CD and UC was 45±13 and 50±27 years, respectively. There was no statistically significant difference in the age between the patients and control participants (P =0.0963). The proportion of males with CD and UC was 38.5% and 54%, respectively. Additional information about the participants is detailed in Appendices XIX and XX.

5.3.4 Sample collection

A total of 350 gastrointestinal biopsies, 48 faecal samples and 51 gum samples were collected during the study (Table 5.1). Routinely, in the collection of biopsies, seven sites were included, which were terminal ileum, caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. In addition four biopsies were collected from stomach of a control participant (AUS35) and three IBD patients (AUS4, AUS10 and AUS36). Three duodenal biopsies were also collected from two IBD patients, (AUS22 and AUS36) and a control participant (AUS35) (Tables 5.1 and 5.2). Some routine biopsies could not be obtained because of the participants' medical condition or surgical removal in the intestine as follows; three biopsies of terminal ileum (two control participants and one UC patient), seven caecal biopsies (CD patients), an ascending colon biopsy (CD patient), a transverse colonic biopsy (CD patient) and a rectal biopsy (control participant) were unavailable. Moreover, four faecal specimens (three IBD and a control participant) were unavailable.

Table 5.1: Numbers of participants and collected specimens in the study.

Patient groups	Number of patients	Biopsy	Stool	Oral Swabs
CD	26 (11M & 15F)	178	26	26
UC	11 (5M & 6F)	75	10	11
Controls	14 (9M & 5F)	97	12	14
Total	51 (25M & 26F)	350	48	51

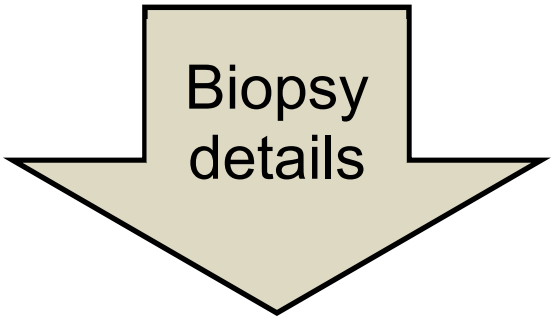


Table 5.2: Number of biopsies collected from each intestinal anatomic site.

Patient groups	TI	Caecum	Ascending Colon	TX	Descending Colon	Sigmoid Colon	Rectum	Duodenum	Stomach	Total
CD	26	20	25	25	26	26	26	1	3	178
UC	9	11	11	11	11	10	11	1	0	75
Healthy	12	14	14	14	14	14	13	1	1	97
Total: 51	47	45	50	50	51	50	50	3	4	350

TI: Terminal ileum. TX: transverse colon. M: male. F: female.

5.3.5 Detection, characterisation and further analysis of *C. concisus* in collected clinical samples

Isolation, detection and characterisation of *C. concisus* in the samples were divided into four sections as follows:

Section I: The prevalence of *C. concisus* in the collected samples was assessed using two techniques: conventional culture method to isolate the live bacterium, and PCR to detect the bacterial DNA in the clinical specimens.

Section II: The prevalence of genomospecies A & B in the obtained *C. concisus* isolates from the clinical samples in section I. In this section also a nested PCR targeting *cjaC* was developed to investigate the prevalence of genomospecies B in the clinical samples where *C. concisus* DNA was detected (in section I).

Section III: The prevalence of *C. concisus* isolates possessing *zot* was investigated. In addition, the prevalence of *zot* in clinical samples (in the extracted DNA of these clinical samples) using the developed nested PCR was assessed. Moreover, PCR products obtained from amplifying *zot* in DNA extracted from selected *C. concisus* isolates were sequenced and analysed.

Section IV: The prevalence of different *C. concisus* groups based on the sequence of *cjaC* and its flanking region. A PCR technique was applied using primer set VI (as described in section 3.3.3.2) to amplify this region from *C. concisus* isolates, which were previously identified to be genomospecies B as this region was found to be heterogonous in this genomospecies. As discussed in chapter 3, this primer set was not successful in amplifying a PCR product in *C. concisus* isolates from genomospecies A.

Section V: SDS-PAGE profiles of whole cell lysates of *C. concisus* isolates obtained from clinical samples of the same patient at the same time were compared (including intestinal biopsies, faeces and gum samples) to further determine the relatedness between isolates.

5.3.5.1 Section I: Detection of *C. concisus*

The suspected 2-4 day old colonies appeared metallic in colour, 1-3 mm in diameter and undulate/rough/entire in shape. It was also noticed that *C. concisus* colonies grown at the same time can infrequently appear in different sizes and colours on HBA. It was observed that heavy mixed growth was fairly common in bacterial cultures from the biopsies and faecal specimens even after the filtration technique as shown in Figures 5.4 and 5.5. Each suspected *C. concisus* colony was subcultured onto HBA prior the confirmation. All *C. concisus* prospective colonies were negative for catalase, unable to grow aerobically and oxidase positive.

A. In biopsies

In a total of 350 biopsies collected from 51 participants, only three *C. concisus* isolates were obtained from three biopsies of three different IBD patients (AUS22, AUS48 and AUS51) (Table 5.3 and Appendix XXI). Two isolates were obtained from 2/75 biopsies of 11 UC patients (the first isolate was obtained by culturing the biopsy directly onto HBA, while the second isolate was obtained by enrichment of the biopsy in Ham's F-12). The third isolate (obtained by enrichment culture of the biopsy in Ham's F-12) was obtained from 1/178 biopsies collected from 26 patients with CD. No *C. concisus* isolates were obtained from 97 biopsies collected from the 14 control participants. The first and second *C. concisus* isolates (AUS22-Bd2 and AUS48-Ba) were obtained from a duodenal biopsy and ascending colonic biopsies of UC patients (AUS22 and AUS48). The third isolate (AUS51-Bd) was obtained from a descending colonic biopsy of a CD patient (AUS51). This is the first reported isolation of *C. concisus* from the duodenum and ascending colon.

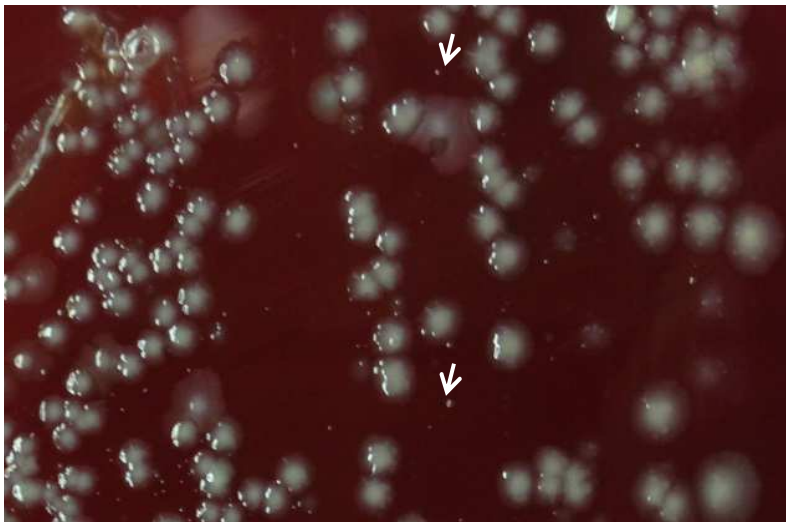


Figure 5.4: Example of mixed bacterial growth obtained from an intestinal biopsy. The biopsy was cultivated onto a HBA plate and incubated in microaerobic condition containing H_2 (the growth condition of *C. concisus*) for 2 days. The small grey colonies (white arrows) were isolated and subjected to further *C. concisus* identification tests.

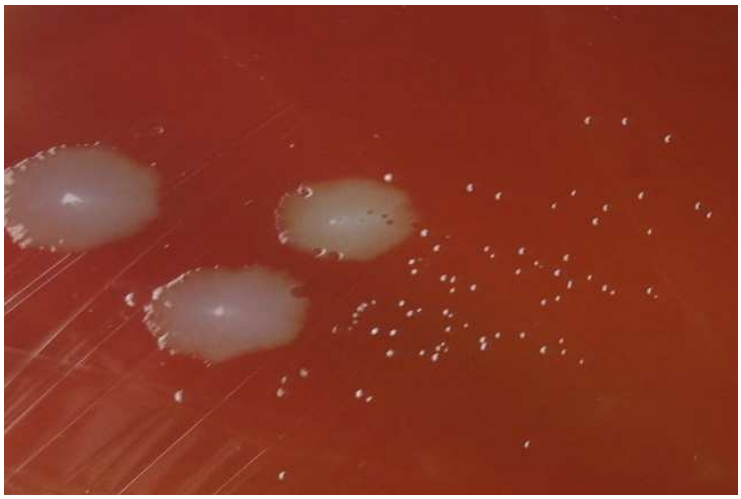


Figure 5.5: Representative mixed bacterial growth obtained from a faecal sample after filtration technique. The sample was cultivated on a HBA plate by filtration technique (2 days incubation in microaerobic condition containing H_2). The small grey colonies were subjected to further tests for *C. concisus* identification.

C. concisus DNA was detected in intestinal biopsies in 29 out of 37 IBD patients (23/26 CD and 6/11 UC patients). In seven IBD patients (AUS1, AUS3, AUS5, AUS6, AUS7, AUS8 and AUS13), the bacterium was detected in all seven intestinal anatomic sites. While *C. concisus* was detected in at least two intestinal anatomic sites in seven patients (AUS4, AUS14, AUS29, AUS36, AUS50, AUS51 and AUS52), it was only detected in a single anatomic site in three patients (AUS9, AUS10 and AUS39). DNA of *Campylobacter* spp. was amplified by PCR step I as stated in section 5.2.9.2 from a biopsy collected from transverse colon of patient AUS46 and it could not be amplified by the second PCR as stated in section 5.2.9.2 PCR step II. *C. concisus* was identified in two biopsies collected from the stomach and duodenum. In general, *C. concisus* could be detected in all of the targeted anatomic sites of the gastrointestinal tract and the detection rate was the highest in the rectum.

In three out of the 14 control participants (AUS11, AUS30 and AUS35) *C. concisus* was detected in at least two intestinal anatomic sites. In addition, none of control participants had the bacterium in all collected biopsies. The highest detection rate of *C. concisus* was in the descending and transverse colon; whereas *C. concisus* could not be detected in the terminal ilium in any of control participants.

B. Faecal samples

By conventional culture techniques of faecal samples, a total of eight *C. concisus* isolates were obtained from 48 faecal specimens. Seven isolates were obtained from three IBD patients and one isolate was obtained from a control participant (1/12); whereas, *C. concisus* could not be isolated from faecal specimens of 11 patients with UC (0/11) (Table 5.3). To our knowledge, this was the first study that isolated *C. concisus* from faeces of adults with IBD. However, when molecular methods (PCR) were used the *C. concisus* DNA was detected in

faecal samples from most of the participants except five IBD patients (three CD and two UC patients) and four control participants.

C. Gum samples

C. concisus was isolated from the majority of the participants and the number of colonies varied between the participants, but in most cases several colonies were obtained from the same participant (1-8 isolates). A total of two hundred and five isolates were obtained from the gum samples of 51 participants. A total of 164 (80 %) *C. concisus* oral isolates were collected from IBD patients (115 isolates from CD and 49 isolates from UC patients) and a total of 41 (20%) *C. concisus* oral isolates were collected from control participants. *C. concisus* was isolated from 86% (32/37) of the gum samples of IBD patients [it was isolated in 85% (22/26) of CD patients and in 91% (10/11) of UC patients], while it was isolated from 64% (9/14) of control participants (Appendix XXI). Therefore, based on these outcomes it seemed that the colonisation of *C. concisus* is higher in IBD patients than in control participants, but the difference was not statistically significant (Table 5.4). Multiple *C. concisus* isolates were obtained from the oral cavity of all the participants except two patients (AUS21 and AUS51) and a control participant (AUS40), which only one isolate was obtained (Appendix XXI). However, when molecular methods (PCR) were used, *C. concisus* DNA was detected in all collected gum samples of IBD patients while it could not be detected in one control participant.

Table 5.3: Proportion of *C. concisus* isolation from intestinal samples collected from IBD patients and control participants by culture technique.

Patient groups	Biopsy isolates	Faeces isolates	All Intestinal isolates
IBD	8% (3/37)	5.7% (2/37)	13.5% (5/37)
Controls	0% (0/14)	7.8% (1/13)	7% (1/14)
Total	**6% (3/51)**	**6.3% (3/48)**	**12% (6/51)**

Table 5.4: Proportion of *C. concisus* isolation from gum samples collected from IBD patients and control participants by culture technique.

Recruits groups	*C. concisus* isolation/participant	*C. concisus* isolates
IBD	86% (32/37)	80% (164/205)
Controls	64% (9/14)	20% (41/205)
Total	**80% (41/51)**	**205**

Overall, the prevalence of *C. concisus* as detected by PCR in intestinal biopsies was significantly higher in IBD patients with a proportion of 59.5% (22/37) than in control participants with a percentage of 21.4% (3/14) (P =0.0073) (Figure 5.6). There was no substantial difference in *C. concisus* detection rate between CD (61.5%; 16/26) and UC patients (54.5%; 6/11). In faeces, *C. concisus* prevalence was 85.3% (29/34) in IBD patients and was 69.2% (9/13) in control participants (P =0.237) (Figure 5.6). In the oral cavity, the bacterium was detected in 100% of patients with IBD, and in 92.9% of control participants, which was not significantly different (Figure 5.6).

The study also looked at the prevalence of *C. concisus* DNA in biopsies that were collected from the seven anatomic sites of the intestinal tract. In all investigated intestinal anatomic sites, *C. concisus* had a higher prevalence in IBD patients than in control participants. The prevalence of *C. concisus* in terminal ilium, sigmoid colon and rectum of IBD patients was significantly higher than in control participants (P =0.0201, 0.0405 and 0.0188, respectively) (Figure 5.7). Although, the prevalence of *C. concisus* in all seven intestinal anatomic sites except the sigmoid colon was higher in CD patients than in UC patients, the difference was not statistically significant. *C concisus* could not be detected in any biopsy collected from the terminal ilium of control participants (Figure 5.7).

There was a statistically significant higher detection rate of *C. concisus* in the biopsies and faecal samples by PCR than by culture technique, while in gum swabs the detection rate of *C. concisus* was not significantly different between the two methods (Figure 5.8). The significant difference could be due to the higher sensitivity of PCR than culture, in particular when the bacterium is fastidious and present in very small numbers. In addition campylobacters could be present in viable but non-culturable form in the intestine.

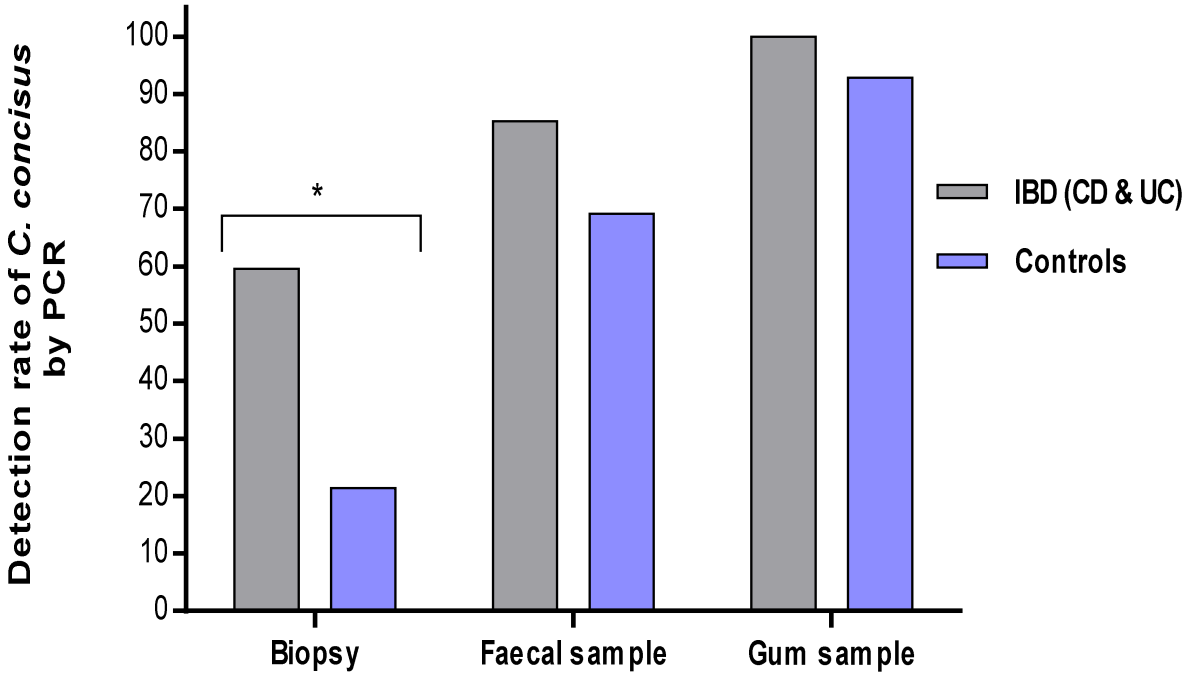


Figure 5.6: Detection rate of *C. concisus* by PCR in collected clinical samples. *C. concisus* DNA was detected in biopsies, faecal samples and gum samples of patients with IBD and control participants using nested PCR technique targeting the 16S rDNA gene. Bars represent *C. concisus* DNA detection rate in at least one of seven biopsies collected from each individual participant, while in faeces and gum swabs *C. concisus* detection was in one sample of each patient. The Fisher's exact test (2-tailed test) was used to calculate *P* values. *: the power of significant difference as compared with control participants (controls) (*P* <0.05). *C. concisus* prevalence in biopsies of IBD patients was significantly higher than in control participants.

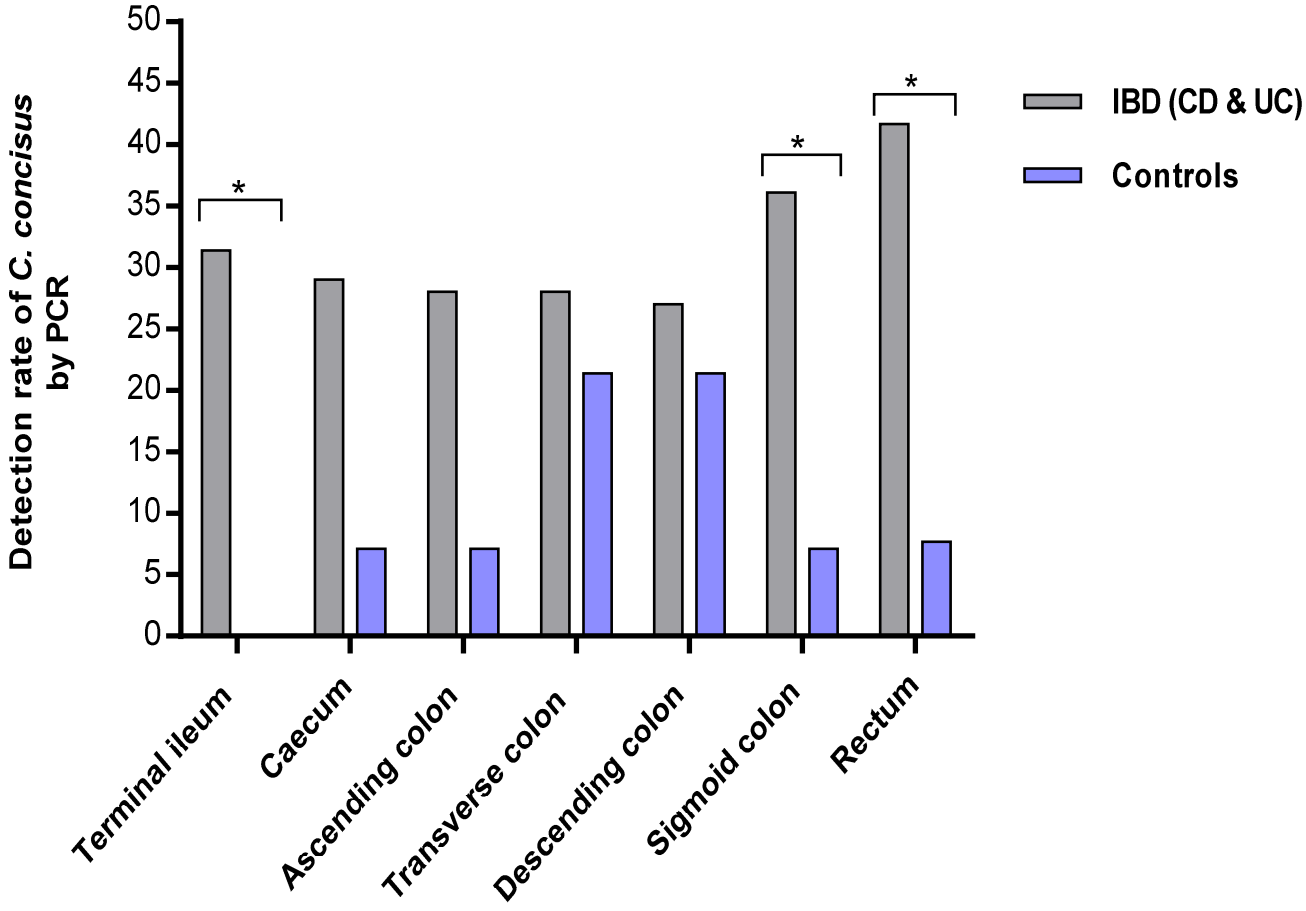


Figure 5.7: Detection rate of *C. concisus* by PCR in biopsies collected from seven intestinal anatomic sites. *C. concisus* DNA was detected directly in intestinal biopsies collected from different anatomic sites of patients with IBD and control participants using nested PCR targeting the 16S rDNA gene. Bars represent *C. concisus* DNA detection rate in at least one biopsy of each individual participant. The Fisher's exact test (2-tailed test) was used to calculate *P* value. Stars are the power of significant difference as compared with control participants (controls) ($P < 0.05$). The prevalence of *C. concisus* in intestinal biopsies collected from terminal ileum, sigmoid colon and rectum of IBD patients were significantly higher than in biopsies collected from the same sites of control participants. *C. concisus* could not be detected in the terminal ileum of control participants.

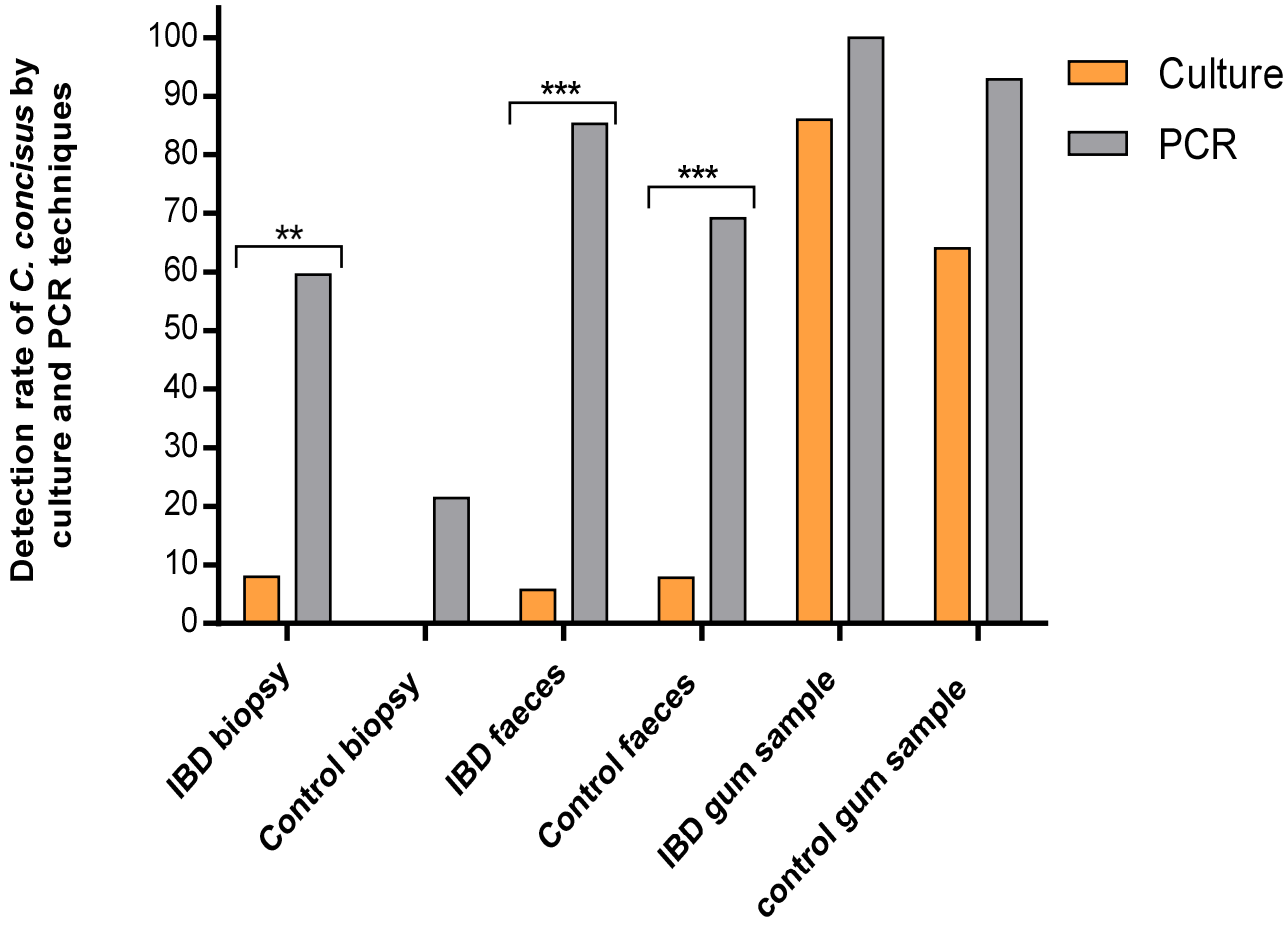


Figure 5.8: A comparison between the detection of *C. concisus* by culture and PCR in the collected clinical samples. *C. concisus* detection was by culture and by PCR in biopsies, faecal samples and gum swabs of patients with IBD and control participants. Culture bars represent *C. concisus* detection rate in any of seven sites of intestinal biopsy, a faecal sample and a gum sample of each individual participant by filtration technique. PCR bars represent DNA detection rate in at least one out of seven biopsies, a gum sample and a faecal sample of an individual participant by nested PCR. The Fisher's exact test (2-tailed test) was used to calculate *P* values. The number of stars are the power of significant difference as compared with control participants (control) (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$). Detection rate of *C. concisus* in biopsies and faecal samples by PCR was significantly higher than culture.

5.3.5.2 Section II: Molecular typing of *C. concisus* isolates and *C. concisus* DNA in clinical samples

A. Genotyping of *C. concisus* isolates

The results showed that the majority of the isolates were from genomospecies B (64%; 132/205). The prevalence of genomospecies B in the oral cavity of IBD patients was significantly higher than in the oral cavity of control participants with a percentage of 71% (116/164) and 39% (16/41) respectively (P <0.0001). The prevalence of genomospecies B was also significantly higher in the oral cavity of IBD patients (in both CD and UC patients) than genomospecies A as shown in Figure 5.9 (P <0.0001). Despite the prevalence of genomospecies A was higher in control participants than genomospecies B, there was no significant difference between the two genomospecies (Figure 5.9). It was found that 23% of CD patients, 36% of UC patients and 21% of control participants were colonised by multiple genomospecies.

The prevalence of genomospecies B isolates obtained from the intestinal specimens (biopsies and faeces) of IBD patients was 54% (11 isolates obtained from six participants). Participant AUS5 (a CD patient) had both genomospecies (six isolates) in faeces, participant AUS22 (a UC patient) had genomospecies A and others (AUS4, AUS47, AUS48 and AUS51) had genomospecies B. Thus, the majority of the participants had genomospecies B in the intestinal tract, as was found for the gum samples. AUS5 had both genomospecies (A & B) in the faeces and the gum while others had either genomospecies A or B as shown in Appendix XXI.

B. Detection and typing of *C. concisus* genomospecies B in clinical samples

A nested PCR was developed to amplify *cjaC* (GB-PCR) and used to investigate the prevalence of genomospecies B in the extracted DNA from biopsies, faecal samples and gum samples. This nested PCR was only applied on the specimens that *C. concisus* DNA was

detected previously in section 5.3.5.1. The primer sets initially were applied on several reference strains including *C. concisus* ATCC 51561, ATCC 51562, RCH3, RCH4, RCH6, RCH11, RCH15, RCH20, RCH26, *C. mucosalis* ATCC 43264 and *E. coli* ATCC 25922 (Table 2.1) to confirm the specific amplification of *C. concisus* genomospecies B DNA as shown in Figures 5.10 (A and B). Then GB-PCR (*cjaC* nested PCR) was applied on the clinical specimens as demonstrated in Figures 5.11 (A and B). To confirm GB-PCR specificity, a random GB-PCR product obtained from biopsy of AUS7 patient (ascending colon) sequence showed identity of >95% to *C. concisus cjaC* gene of *C. concisus* 13826.

Genomospecies B was detected in 35.1% (13/37) of patients with IBD (Figure 5.12). The highest prevalence was in descending colon, while the lowest prevalence was in transverse colon and ascending colon. Furthermore, genomospecies B DNA was detected in the two stomach biopsies collected from participants AUS4 and AUS36 respectively. In AUS36 the genomospecies was also detected in a duodenal biopsy. Genomospecies B was identified in all the gum samples of IBD patients, while it was detected in 55.9% of the faecal specimens of those patients (Figure 5.12).

In the control participants, genomospecies B DNA was identified in 1/14 (7%, AUS35) biopsies. In addition, this genomospecies DNA was detected in 78.6% (11/14) in gum samples, while in faecal specimens, 23.1% (3/13) of the participants had the genomospecies B DNA (Figure 5.12).

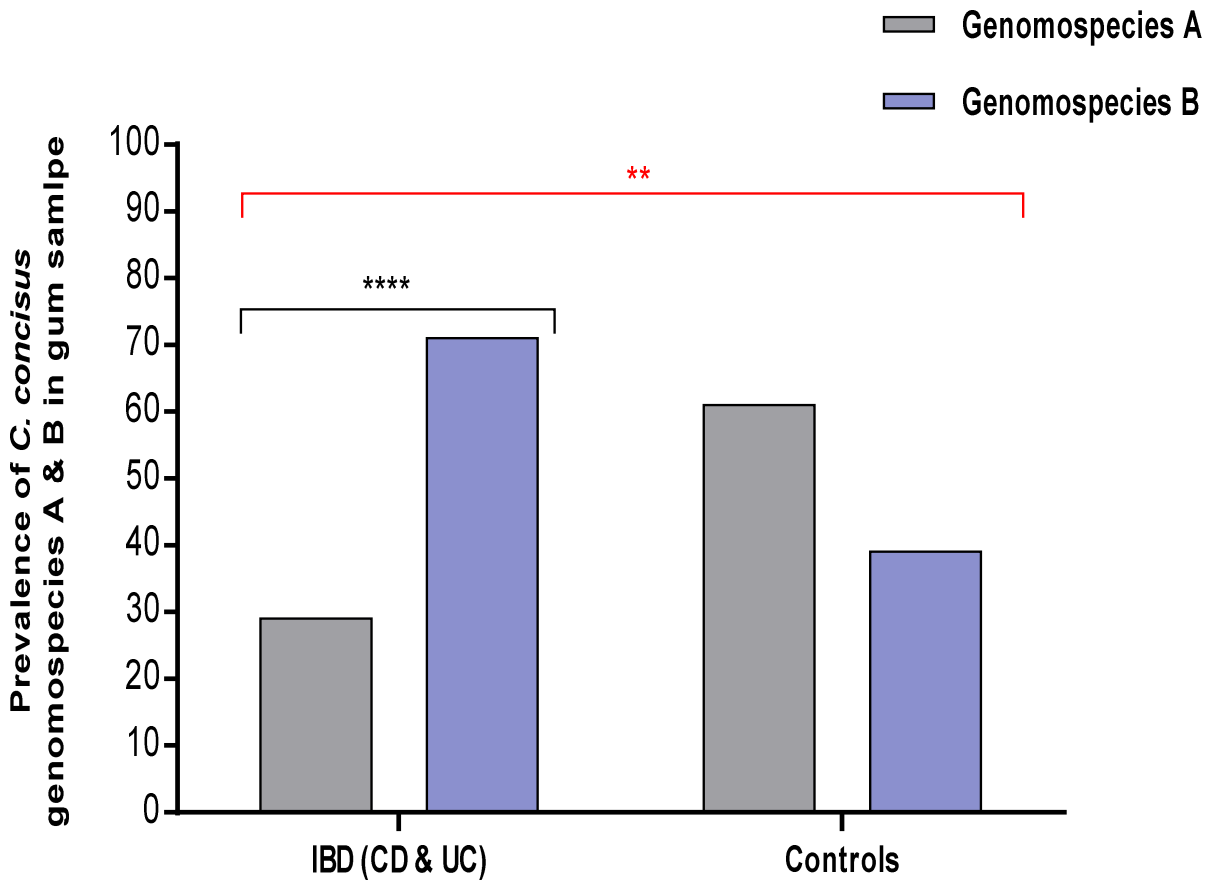
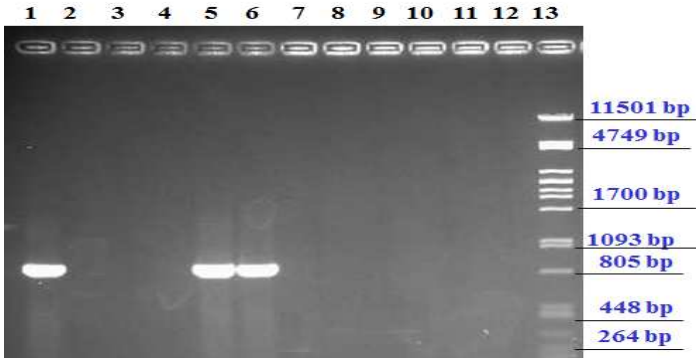


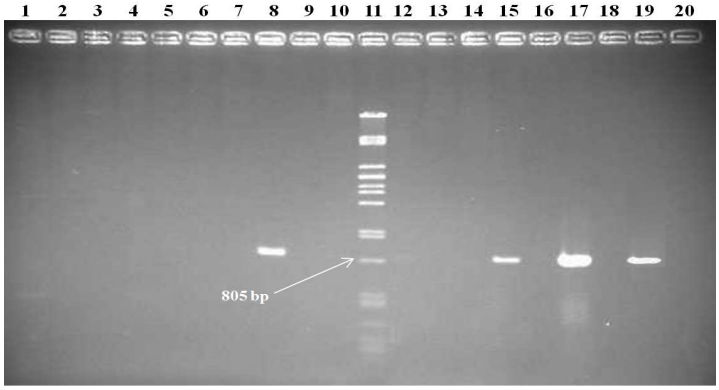
Figure 5.9: Prevalence of genomospecies A and B in *C. concisus* isolates obtained from gum samples. Bars are *C. concisus* genomospecies detected by amplifying the 23S rDNA gene (MUC1 and CON1/CON2) from *C. concisus* isolates obtained from gum samples of IBD patients and control participants. The statistical analysis calculation (presented as stars in black colour) was based on the comparison between the prevalence of genomospecies A and genomospecies B strains in IBD patients, while the statistical analysis calculation (presented as stars in red colour) was based on the comparison between the prevalence of genomospecies A or B strains in IBD patients and in control participants (controls). Fisher's exact test (2-tailed test) was used to calculate *P* values. Stars indicate the level of significant difference between genomospecies A and genomospecies B (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$). The prevalence of genomospecies B isolates was significantly higher than genomospecies A isolates in IBD patients ($P < 0.0001$) whilst the prevalence of genomospecies A isolates was higher than genomospecies B isolates in control participants, but it was not statistically significant differences. In addition, the prevalence of genomospecies A isolates was significantly higher in control participants than in the IBD patients; however, the prevalence of genomospecies B isolates was significantly higher in IBD patients when compared to the control participants.



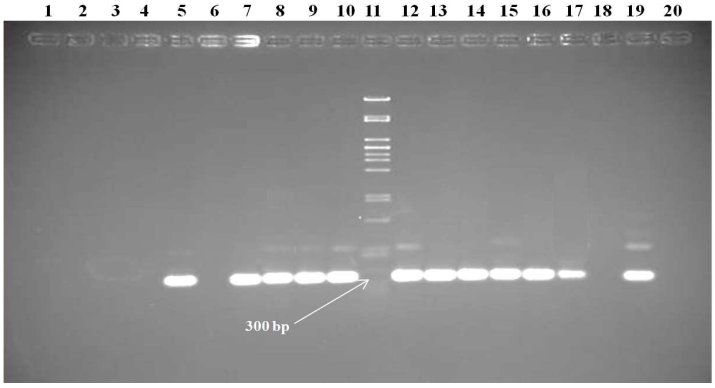
(A)

(B)

Figure 5.10: Application of *cjaC* by the developed *cjaC* nested PCR for typing *C. concisus* genomospecies B reference strains. The PCR products obtained from amplifying *cjaC* from genomic DNA as a template. In (A), PCR products (800 bp) were obtained from the amplification of *cjaC* by the first step nested PCR using primer set I. In (B), PCR products (300 bp) were obtained from the amplification of the first step PCR product using primer set II. PCR products were loaded on a 1.5% (w/v) agarose gel for electrophoresis. The gel was then stained in ethidium bromide and visualised by a UV trans-illuminator. Lanes 1-9: *C. concisus* strains ATCC 51561, ATCC 51562, RCH3, RCH4, RCH6, RCH11, RCH15, RCH20 and RCH26, respectively. Lanes 10-12: *C. mucosalis* (ATCC 43264), *E. coli* **(**ATCC 25922) and no template control. Genomospecies A strains were ATCC 51562, RCH3, RCH4, RCH15, RCH20 and RCH26, while genomospecies B strains were ATCC 51561, RCH6 and RCH11. Lane 13: lambda DNA marker.



(A)



(B)

Figure 5.11: Detection of *C. concisus* genomospecies B in clinical samples using the *cjaC* nested PCR. The PCR products obtained from amplifying *cjaC* from the clinical samples. In (A), PCR products (800 bp) were obtained from the amplification of *cjaC* by the first step nested PCR using primer set I. In (B), PCR products (300 bp) were obtained from the amplification of the first step PCR product using primer set II. PCR products were loaded on a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. Lanes 1-9: AUS7 specimens (biopsies of terminal ileum, caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum; then, the faecal and gum samples). Lanes 10, 12, 13, 14, 15, and 16: AUS8 specimens (biopsies of terminal ileum, descending colon, sigmoid colon and rectum; then the faecal and gum samples). Lanes 17-20: ATCC 51561, ATCC 51562, RCH11, and no template control. Lane 11: lambda DNA marker.

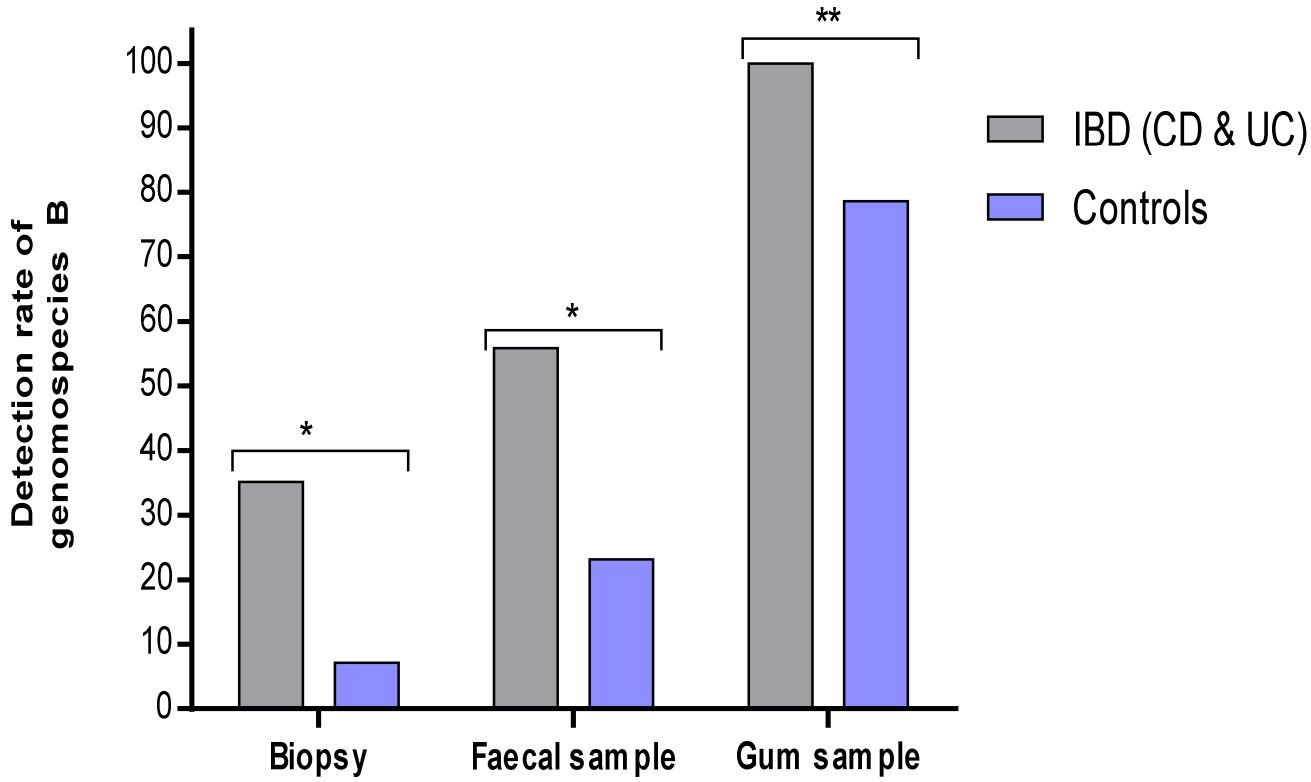


Figure 5.12: Detection rate of *C. concisus* genomospecies B DNA in clinical samples by the *cjaC* nested PCR. Bars represent the detection rate of *C. concisus* genomospecies B in extracted DNA from biopsies, faecal samples and gum samples collected from patients with IBD (CD and UC) and control participants (controls) by the developed nested PCR to amplify *cjaC*. The Fisher's exact test (2-tailed test) was used to calculate *P* values. The number of stars are the power of significant difference of genomospecies B detection in IBD patients compared to the control participants ($P < 0.05$). The prevalence of genomospecies B in all clinical samples (biopsies, gum samples and faecal samples) of IBD patients was significantly higher than in the control participants.

In summary, in section II genomospecies B isolates were predominant in all clinical samples of IBD patients, while in control participants genomospecies A isolates were predominant. In the oral cavity of IBD patients, the isolation of *C. concisus* strains belonging to genomospecies B was significantly higher than genomospecies A (P <0.0001). In contrast, the majority of oral isolates from the control participants were from genomospecies A (P =0.0766). Moreover, the detection of genomospecies B DNA in the clinical samples showed that the prevalence of genomospecies B was significantly higher in IBD patients than in the control participants, [biopsies (P =0.0456), faeces (P =0.0438) and gum samples (P= 0.003)]. Therefore both techniques demonstrated that in all clinical samples of IBD patients, genomospecies B was present in a higher prevalence than in control participants, which was statistically significant indicating the possible association between genomospecies B and IBD.

5.3.5.3 Section III: Detection of *zot* in *C. concisus* isolates and in clinical samples

A. Detection of the *zot* gene in *C. concisus* using one step PCR

A PCR technique described in section 3.2.5 was performed to amplify *zot* in the *C. concisus* isolates (Kalischuk *et al.* 2011). The gene was only amplified from eight isolates obtained from the gum samples of five participants (3 IBD patients and 2 controls) (Table 5.5). The gene could not be amplified from the intestinal isolates. The majority of the isolates (75%, 6/8) that possessed *zot* belonged to genomospecies A (Table 5.5). Overall, the proportions of *C. concisus* isolates that had *zot* in IBD patients and control participants were 3.1% (5/164) and 7.3% (3/41) respectively, which were not statistically significant.

B. Detection of *zot* in clinical samples by nested PCR

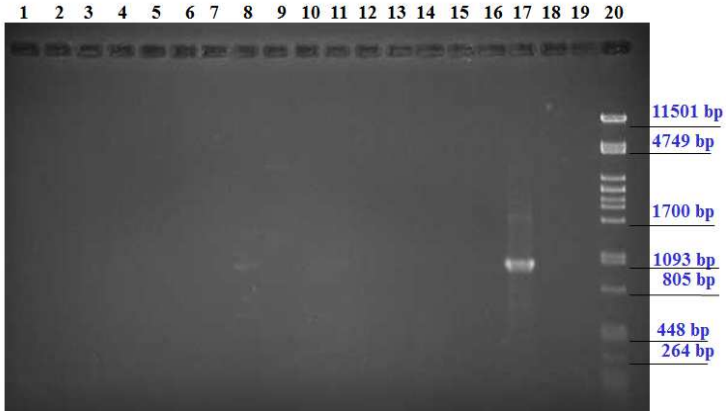
The nested PCR was applied to all clinical samples for which *C. concisus* DNA could be amplified (section I), an example for *zot* amplification in biopsies, faecal and gum samples by

nested PCR is shown in Figure 5.13 (A and B). The detection rate of *zot* in the clinical samples was 45.1% (23/51): 12.1% (11/91) of the biopsies, 40% (20/50) of gum samples and 23.7% (9/38) of the faecal samples. There was no significant difference in the detection rate of *zot* in biopsies of IBD patients (8.1%) and control participants (7.1%). In addition, there was no significant difference in the detection rate of *zot* in gum samples of IBD patients (37.8%) and of the control participants (42.9%), (Figure 5.14).

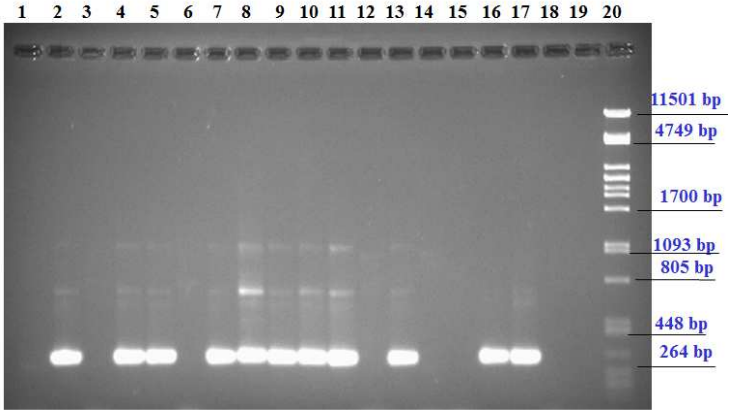
As *C. concisus* possessing *zot* could not be isolated from biopsies or faecal samples, a comparison in the detection rate of *zot* was performed between the culture and PCR techniques in the gum samples. The detection rate of *zot* in the gum samples of IBD patients by PCR (37.8%) was significantly higher than the detection rate by culture technique (8%) (P =0.0047). The detection rate of *zot* in the gum samples of the control participants by PCR was higher than by culture technique, but the difference was not statistically significant (P =0.6351 and P =0.2087, respectively) (Figure 5.15).

Table 5.5: *C. concisus* isolates obtained from gum samples possessing *zot*.

Isolates no.	G. species	Recruit group
AUS7-Ga	A	**UC**
AUS23-Gc	B	**CD**
AUS23-Gd	A	**CD**
AUS26-Ga	B	**Control**
AUS31-Ga	A	**UC**
AUS31-Gb	A	**UC**
AUS47-Ga	A	**Control**
AUS47-Gb	A	**Control**
Total	**8 isolates**	**5 recruits**



(A)



(B)

Figure 5.13: Detection of *zot* in clinical samples using the *zot* nested PCR. The clinical samples were intestinal biopsies, gum samples and faecal samples. In (A) the amplification of *zot* from extracted DNA of clinical samples by the first step nested PCR using primer set ZoT-F/ZotA2-R to amplify 1055 bp. In (B) the amplification of *zot* from the first step PCR by the second step nested PCR to amplify 300 bp of *zot* using primer set ZotB. PCR products were loaded on a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. Lanes: 1-9 AUS7 specimens (biopsies of terminal ileum, caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum; then the faecal sample and gum sample). Lanes: 10-15: AUS8 specimens (biopsies of terminal ileum, descending colon, sigmoid colon and rectum; then the faecal sample and gum sample). Lane16: gum sample of AUS9. Lane 17: RCH6. Lanes 18 and 19: ATCC 51561 and no template control. Lane 20: lambda DNA marker.

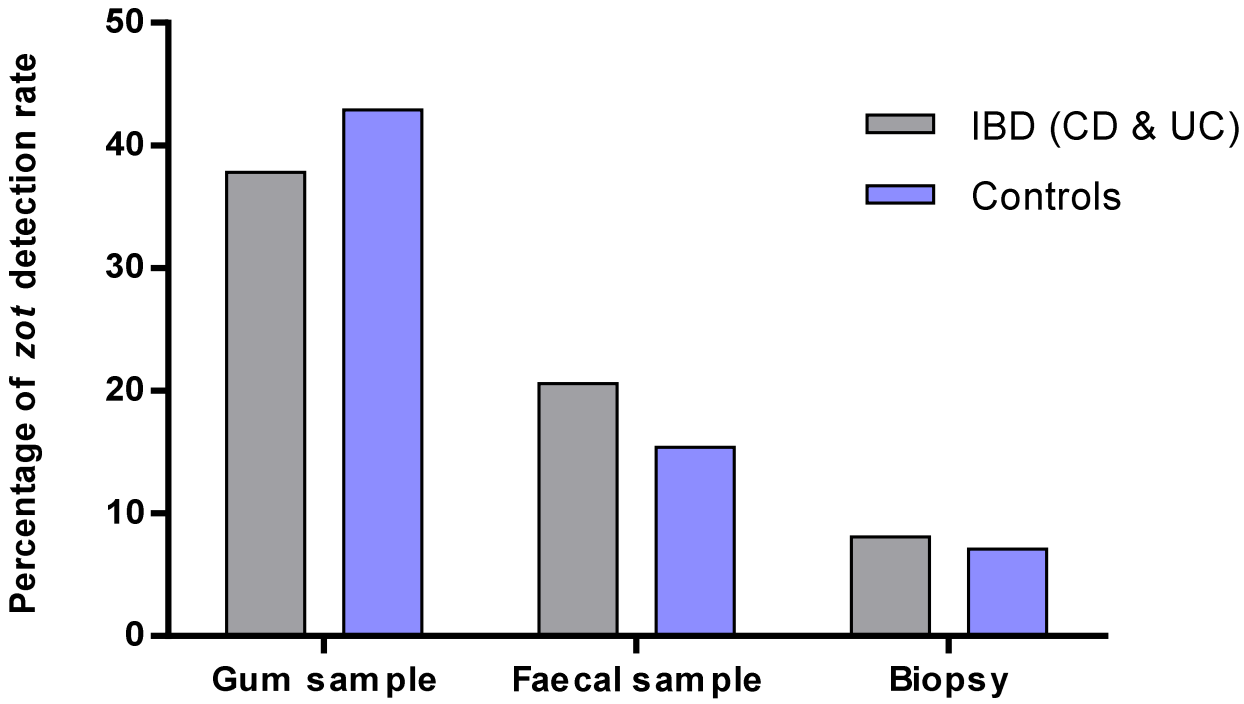


Figure 5.14: Detection rate of *zot* directly in the clinical samples by the *zot* nested PCR. Bars represent detection rate of *zot* in the extracted DNA from the clinical samples. The clinical samples were biopsies, faecal samples and gum samples collected from patients with IBD (CD and UC) and the control participants (controls). The data shows that there is no significant difference in the detection rate of *zot* between the IBD patients and the control participants in any of the clinical sample.

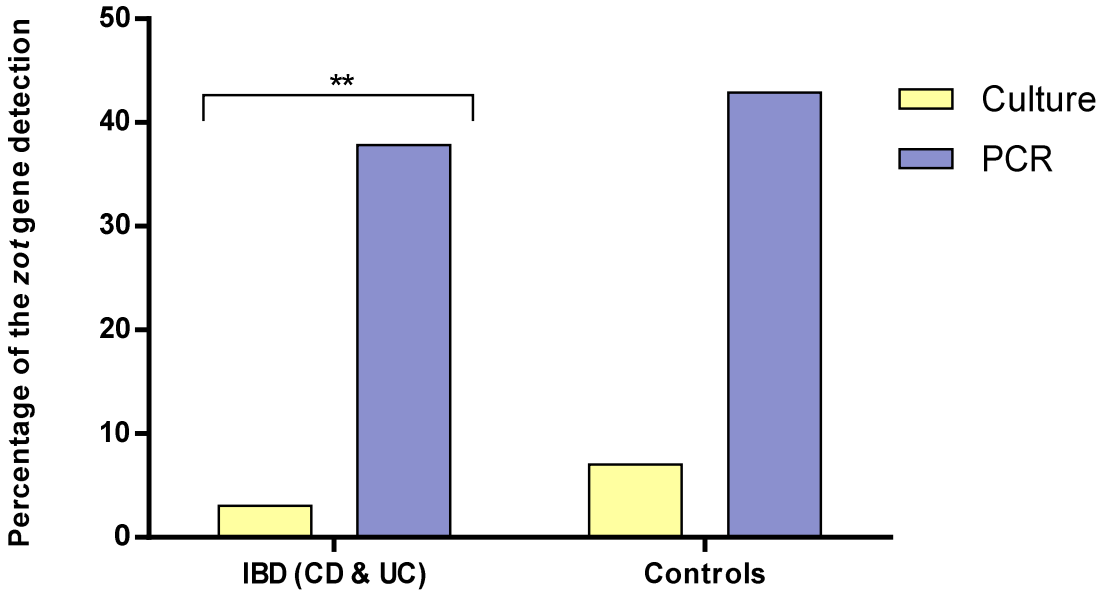


Figure 5.15: A comparison between the detection rate of *zot* directly in gum samples by PCR and in isolates obtained by culture of gum samples. Bars represent detection rate of *zot* in *C. concisus* isolates obtained from gum samples and directly in the gum samples by PCR. The gum samples were collected from patients with IBD (CD and UC) and control participants (controls). The Fisher's exact test (2-tailed test) was used to calculate *P* values. The number of stars are the power of significantly different detection rates between culture and PCR techniques ($P < 0.05$). In gum samples of IBD patients, there is a significant difference between the detection rate of *zot* by PCR directly from the samples and cultured *C. concisus*.

C. Sequence analysis of the *zot* gene from *C. concisus* isolates

One oral isolate from each participant was selected with the exception of two isolates belonging to different genomospecies (A and B) were obtained from participant AUS23. The PCR products of *zot* gene obtained from four isolates (AUS7-Ga, AUS23-Gc, AUS23-Gd and AUS26-Ga) were sequenced and used in the analysis. The PCR products from RCH6, RCH11 and RCH26 (previously detected to possess the gene in section 3.3.4) as well as from mention above isolates, which also possessed *zot* were sequenced.

Useable sequence was obtained from a 966 bp region of *zot* gene for each of the *C. concisus* isolates. The sequenced region (5´-3´) started at locus number 91 and terminated at position number 1056 based on published *C. concisus* 13826 sequence. Of seven other isolates available via WGS on the NCBI data-base site only UNSW3 and UNSWCS were found to possess *zot*. Alignment of the 966 bp of the *zot* sequence was performed using Clone manager suite software and the clinical isolates were compared to the sequence of the published strains (*C. concisus* 13826, UNSWCS and UNSW3). The results of this alignment are shown in Appendix XXII. From the ten isolates, two (13826 and RCH6) had identical sequences and the identity for another two was ≥94% (AUS23-Gc; 97% and AUS26-Ga; 94%). The identity for the remaining six isolates was between 87%-89%. There were at least 75 positions in *zot* where there were nucleotide substitutions in more than two strains (Appendix XXII).

The identity of *zot* sequences in genomospecies A and B was investigated in seven isolates from genomospecies B (13826, UNSWCS, UNSW3, RCH6, RCH11, AUS23-Gc and AUS26-Ga) and three isolates (RCH26, AUS7-Ga and AUS23-Gd) belonging to genomospecies A. The identity was higher between the isolates from genomospecies A (>94%) than those belonging to genomospecies B (>88%).

The amplified nucleotide sequences of *zot* from ten isolates were also compared by the neighbour-joining dendrogram (Figure 5.16). Two main groups (I and II) could be generated

by this method (Figure 5.16). Each group was classified into two subgroups (Ia, Ib, IIa and, IIb) based on the generated clades. Group I included genomospecies B and genomospecies A isolates; however, genomospecies B isolates (UNSWCS, RCH11 and UNSW3) formed subgroup Ia, while all genomospecies A (RCH26, AUS7-Ga and AUS23-Gd) were allocated in subgroup Ib. Other genomospecies B isolates (13826, RCH6, AUS26-Ga and AUS23Gc) were allocated to group II. Surprisingly; subgroup IIa consisted of only one isolate from the gum of a control participant (AUS26-Ga) (Figure 5.16).

The nucleotide sequence of *zot* in the ten tested *C. concisus* isolates were translated into amino acid sequences and aligned to investigate whether the nucleotide polymorphisms had caused major changes in the protein sequence. The DNA sequence (966 bp) resulted in translation of 322 amino acids (from amino acid number 24-348). Amino acid sequence variation was observed between all test isolates and *C. concisus* 13826 reference strain except that the sequence of RCH6 was identical to that of *C. concisus* 13826. The other isolates identities ranged from 89%-99% regardless of whether they belonged to genomospecies A or B. Amino acid changes occurred at 4 to 35 loci per isolate in compared with *C. concisus* 13826 sequence as presented in Table 5.6.

A neighbour-joining dendrogram based on the amino acid sequences were showed similar results to those obtained by DNA sequence analysis; all isolates were assembled into two main groups and each group into two subgroups (Figure 5.17).

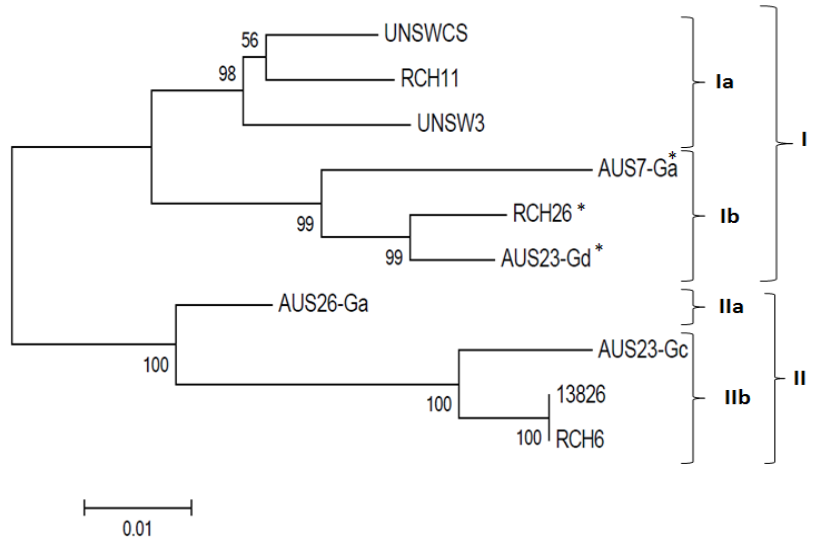


Figure 5.16: Neighbour-joining dendrogram based on *zot* nucleotide sequences of *C. concisus* isolates. UNSW3, UNSWCS and 13826 are *C. concisus* whole genome sequenced strains (Accession No. ANNE00000000, ANNG00000000 and CP000792.1). RCH6, RCH11 and RCH26 are faecal isolates from children with gastroenteritis. AUS7-Ga, AUS23-Gc and AUS23-Gc are oral isolates from patients IBD. AUS26-Ga is an oral isolate form a control participant. *: genomospecies A isolates. I and II: *C. concisus* isolates groups generated by the dendrogram. a and b refer to subgroups generated by the dendrogram.

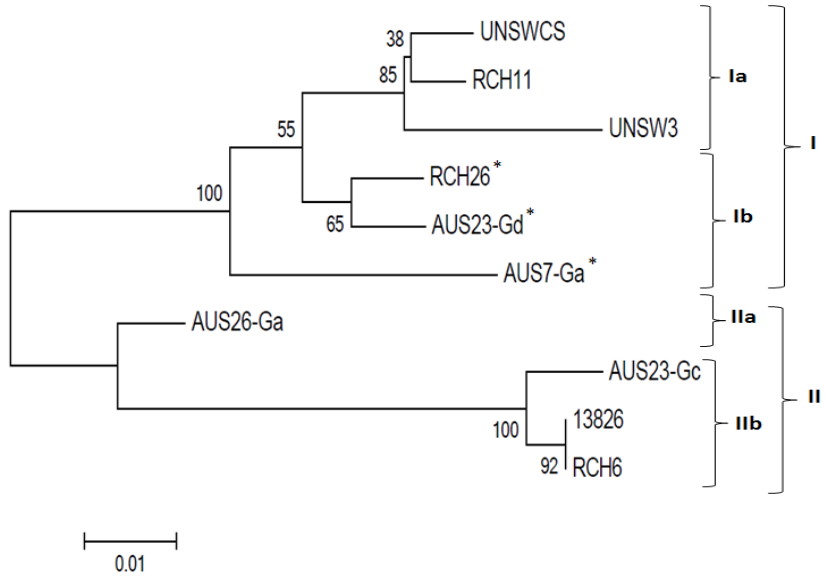


Figure 5.17: Neighbour-joining dendrogram based on amino acid sequences of *zot* in *C. concisus* isolates. UNSW3, UNSWCS and 13826 are *C. concisus* whole genome sequenced strains (Accession No. ANNE00000000, ANNG00000000 and CP000792.1). RCH6, RCH11 and RCH26 are faecal isolates from children with gastroenteritis. AUS7-Ga, AUS23-Gc and AUS23-Gc are oral isolates from patients IBD. *: genomospecies A isolates. I and II: *C. concisus* isolates groups generated by the dendrogram. a and b refer to subgroups generated by the dendrogram.

Table 5.6: Alignment of the amino acid sequences of *zot* in oral, faecal and reference *C. concisus* strains versus *C. concisus* 13826.

Strain	Isolate source	Disease	Genom. species	Amino acid (aa) changes no.			
				Similar aa	Mismatch aa	Total Loci no.	Identities %
UNSW3	Intestine	CD	B	14	21	35	89
UNSWCS	Intestine	gastroenteritis	B	14	20	34	89
RCH6	Intestine	gastroenteritis	B	0	0	0	100
RCH11	Intestine	gastroenteritis	B	14	19	33	90
RCH26	Intestine	gastroenteritis	A	15	19	34	89
AUS7-Ga	Gum	UC	A	15	19	34	89
AUS23-Gc	Gum	CD	A	3	1	4	99
AUS23-Gd	Gum	CD	B	15	19	34	89
AUS26-Ga	Gum	Control	B	7	10	17	95

UNSW3, UNSWCS and 13826 are *C. concisus* strains, which their whole genome sequences are available in the NCBI data-base site under Accession No. ANNE00000000, ANNG00000000 and CP000792.1. RCH6, RCH11 and RCH26 are faecal isolates from children with gastroenteritis. AUS7-Ga, AUS23-Gc and AUS23-Gc are oral isolates from IBD patients. AUS26-Ga is an oral isolate from a control participant.

In summary, the DNA and amino acid sequence analysis of *zot* revealed that the gene was extremely heterogeneous, which may have an effect on the protein stracture. The differences in the sequences were mainly linked to genomospecies type. One isolate that possessed *zot* was from a control participant. The isolate occupied a unique phylogenetic position (subgroups IIa) when compared to the other clinical isolates, possibly resulting in a different gene product compared to IBD patient isolates.

5.3.5.4 Section IV: Detection of *cjaC* and its flanking region in genomospecies B isolates

As the majority of *C. concisus* isolates detected in clinical samples were from genomospecies B (Figure 5.9), a PCR was used to type the isolates. Primer set VI was able to type *C. concisus* isolates from genomospecies B into three different groups as follows: in group I isolates, the PCR product size was 600 bp (as predicted) while in group II isolates, the PCR product size was 1700 bp. In group III (majority of the isolates), there was no PCR product.

The prevalence of group II isolates was significantly higher in control participants than in patients IBD (P <0.002), but there was no significant differences in the prevalence of groups I and III between control participants and patients IBD (Figure 5.18). This finding supports the previous results in section 3.3.3.2 showing that group II could be detected only in an oral isolate (O11) obtained from a healthy individual. Therefore, group II isolates could be more likely to colonise the oral cavity of healthy volunteers and might be good candidates to be used as *C. concisus* non-aggressive isolates in laboratory experiments.

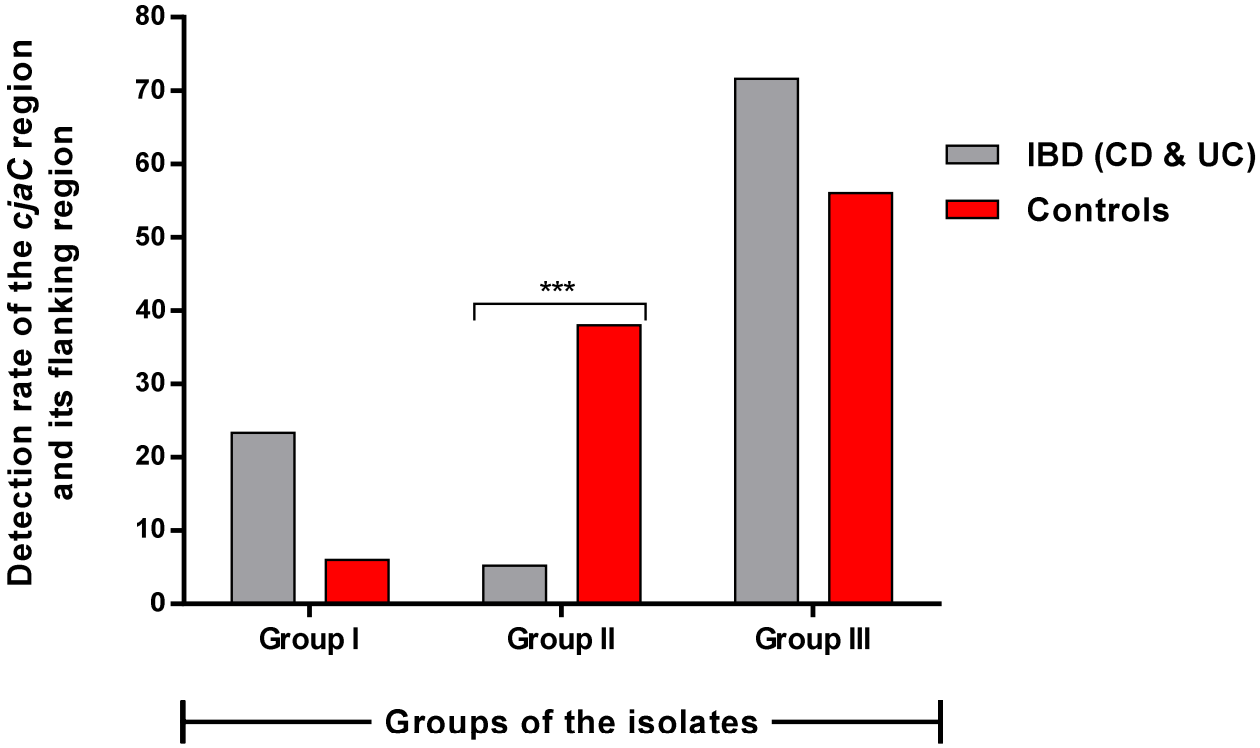


Figure 5.18: Screening of *cjaC* and its flanking region in genomospecies B isolates obtained from gum samples. The detection rate of *C. concisus* isolates groups generated from amplifying the *cjaC* gene and its flanking region (CCC13826_0962). The bars are the detection rate of the *cjaC* gene and its flanking region by PCR using primer set VI in *C. concisus* isolates obtained from gum samples collected from patients with IBD (CD and UC) and control participants (controls). The number of stars are the power of significant difference in the patients as compared to control participants (<0.05). Group I: the region was amplified from the isolates DNA with a size of 600 bp. Group II: the region was produced from the isolates DNA with a large size of 1700 bp. Group III: the region could not be amplified from the isolates DNA. The prevalence of group II in control participants was significantly higher than in IBD patients.

5.3.5.5 Section V: Investigation of the similarity between oral and intestinal *C. concisus* isolates obtained from the same participant using SDS-PAGE

C. concisus was isolated from both gum and intestinal samples of six participants (11.6%) (Table 5.7 and Appendix XXI). The similarity between the isolates from the same participant was investigated using the whole cell lysates protein (WCLP) profile analysis using SDS-PAGE. Thirty-nine bacterial isolates (22 oral and 11 intestinal) and six reference strains, including two *C. concisus* strains (ATCC51561 and 51562), three other campylobacters (*C. mucosalis* ATCC 43264, *C. jejuni* 81116, *C. coli* NCTC 11366) and *E. coli* ATCC 25922 were used in this study (Table 5.7).

All seven *C. concisus* isolates obtained from AUS4 (an IBD patient) showed identical WCLP patterns. This result may indicate that AUS4-Sf (isolated from faeces) translocated from the oral cavity to the gastrointestinal tract (Figure 5.19). Similar results were shown by WCLP patterns of *C. concisus* isolates obtained from AUS5 (an IBD patient) as the oral and faecal isolates showed identical WCLP patterns (Figure 5.20). On the other hand, the *C. concisus* isolates from intestinal biopsies of AUS22 (an IBD patients), AUS48 and AUS51 showed different WCLP patterns from the oral isolates, although they belonged to the same genomospecies [Figure 5.21 (lane 6) and Figure 5.22 (lanes 5 and 7)]. In AUS47 (a control participant), WCLP patterns of each isolate showed distinguishable patterns although all belonged to the same genomospecies (Figure 5.23).

WCLP analysis was performed for bacterial reference strains (*C. concisus* ATCC 51561, ATCC 51562, *C. mucosalis* ATCC 43264, *C. jejuni* 81116, *C. coli* NCTC 11366 and *E. coli* ATCC 25922) to visualise the variations through *C. concisus* strains (genomospecies A and B) and, common isolated bacteria from the gastrointestinal tract including other campylobacters and *E. coli*. All the bacterial strains demonstrated unique WCLP patterns (Figure 5.24). None of the WCLP patterns of *C. concisus* clinical isolates matched WCLP

patterns of bacterial species other than *C. concisus.* The highest variable area for the WCLP in the gels was located between 25-50 KD.

Collectively, *C. concisus* isolates from biopsies showed unique WCLP patterns when compared to the orals isolates. WCLP patterns of faecal isolates were generally similar to these at the oral isolates of the same patients (AUS4 and AUS5), except for isolates from AUS47 (a control participant). It is interesting that the WCLP patterns of the faecal isolates was very similar to WCLP patterns of ATCC 51561 strain (previously isolated from the faecal sample of a healthy control). It is thus possible that isolates from biopsies could produce certain proteins that facilitate their colonisation in the intestinal tract.

It is noteworthy that isolating *C. concisus* from the oral cavity was not linked to the ability to isolate it from intestinal sites as in some participants one isolate yielded from the gum sample and biopsy. However in other participants, several *C. concisus* colonies obtained from the gum sample, but *C. concisus* could not be isolated from the biopsies or faeces. In addition *C. concisus* isolates that yielded PCR products with primer set VI often demonstrated individual protein patterns, for example AUS47-Gc belonged to group I, while AUS51-Bd fitted into group II. Further work needs to be done to include more *C. concisus* isolates belonging to each group based on using primer set VI, to investigate whether they have the same WCLP patterns or different patterns.

Table 5.7: *C. concisus* isolates from intestinal and oral samples of the same participant and used in whole cell protein profile analysis.

Patient code	Isolates	Source	G.S	Patient code	Isolates	Source	G.S
AUS4	AUS4-Ga	Gum	B	**AUS22**	AUS22-Ga	Gum	B
(UC)	AUS4-Gc	Gum	B	**(UC)**	AUS22-Gb	Gum	A
	AUS4-Gk	Gum	B		AUS22-Gc	Gum	B
	AUS4-Gg	Gum	B		AUS22-Gd	Gum	B
	AUS4-Gm	Gum	B		AUS22-Gf	Gum	B
	AUS4-Gn	Gum	B		AUS22-Bd2	Biopsy	A
	AUS4-Sf	Faeces	B	**AUS47**	AUS47-Ga	Gum	A
AUS5	AUS5-Ga	Gum	A	**(Control)**	AUS47-Gb	Gum	A
(CD)	AUS5-Gc	Gum	A		AUS47-Gc	Gum	B
	AUS5-Gd	Gum	B		AUS47-Sa	Faeces	B
	AUS5-Ge	Gum	B	**AUS48**	AUS48-Ga	Gum	B
	AUS5-Sa	Faeces	A	**(UC)**	AUS48-Gb	Gum	B
	AUS5-Sc	Faeces	A		AUS48-Gd	Gum	B
	AUS5-Sf	Faeces	A		AUS48-Ge	Gum	B
	AUS5-Sd	Faeces	B		AUS48-Ba	Biopsy	B
	AUS5-Sb	Faeces	B	**AUS51**	AUS48-Ga	Gum	B
				(CD)	AUS51-Bd	Biopsy	B

G.S: genomospecies.

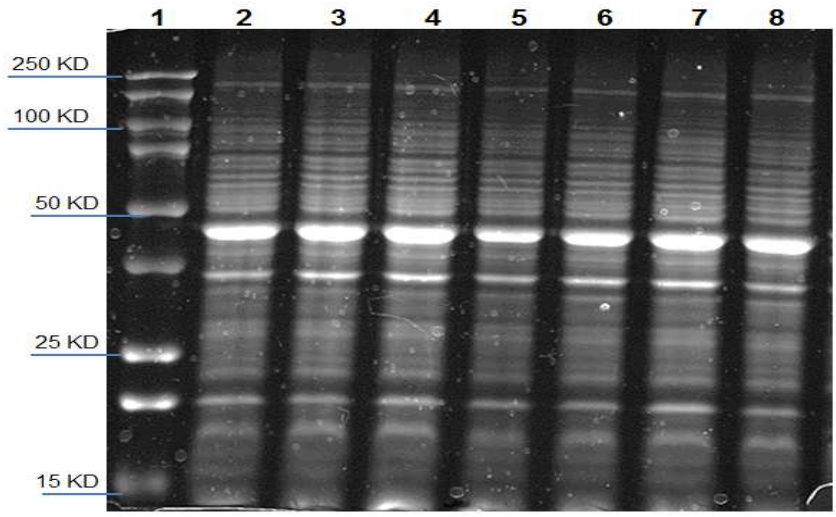


Figure 5.19: SDS-PAGE for whole cell protein profiles of *C. concisus* isolates from gum and faecal samples collected from patient AUS4. The gel was visualised by Coomassie blue stain. Lane 1: protein marker. Lanes 2-8: *C. concisus* isolates (AUS4-Ga, AUS4-Gc, AUS4-Gk, AUS4-Gg, AUS4-Gm, AUS4-Gn and AUS4-Sf, respectively). All the isolates were obtained from gum samples except lane 8, which was from faeces. The isolates were obtained from patient with CD. All the protein profiles of the oral and faecal isolates were identical.

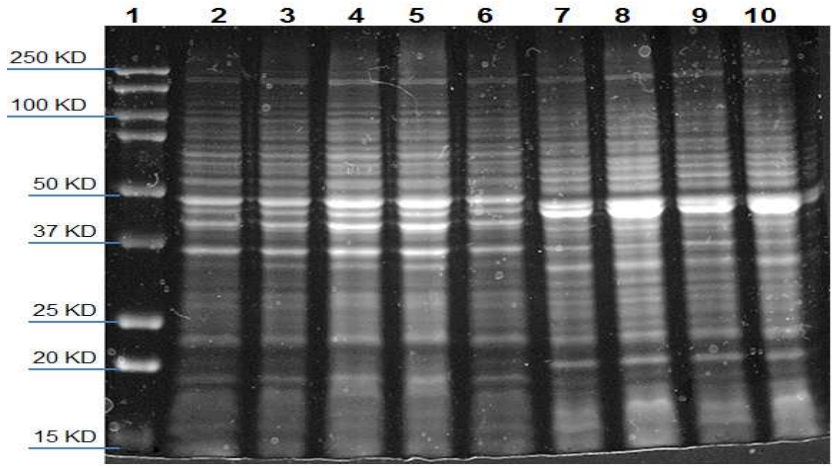


Figure 5.20: SDS-PAGE for whole cell protein profile of *C. concisus* isolates obtained from a gum and faecal samples collected from patient AUS5. The gel was visualised by Coomassie blue stain. Lane 1: protein marker. Lanes 2-10: *C. concisus* isolates (AUS5-Ga, AUS5-Gc, AUS5-Sa, AUS5-Sc, AUS5-Sf, AUS5-Gd, AUS5-Ge, AUS5-Sb and AUS5-Sd, respectively). Lanes 2-6: *C. concisus* isolates belong to genomospecies A, while lanes 7-10: *C. concisus* isolates belong to genomospecies B. Lanes 4, 5, 6, 9 and 10: *C. concisus* isolates from faecal samples, while other lanes were isolated from the gum samples. The isolates were from CD patient. Each faecal isolate showed an identical WCLP patterns to at least one of oral isolate. The differences in WCLP patterns can only be seen between genomospecies A and B.

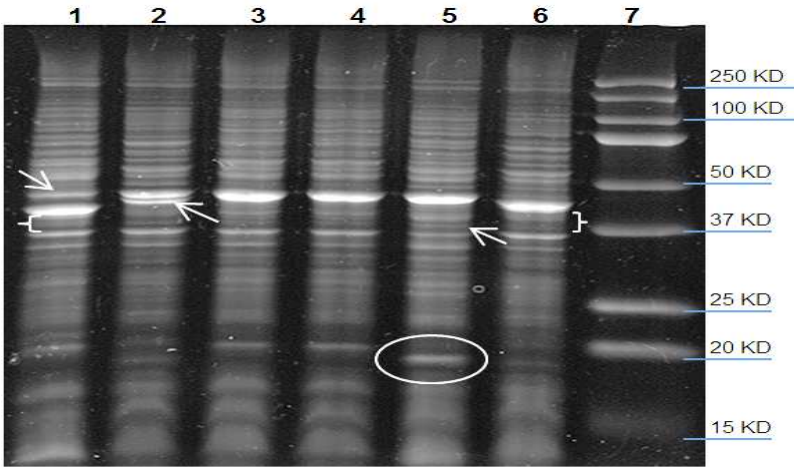


Figure 5.21: SDS-PAGE for whole cell protein profiles of *C. concisus* isolates from a gum sample and biopsy (duodenum) collected from patient AUS22. The gel was visualised by Coomassie blue stain. Lanes 1-6: *C. concisus* isolates (AUS22-Ga, AUS22-Gb, AUS22-Gc, AUS22-Gd, AUS22-Gf and AUS22-Bd2 respectively). Lane 6: *C. concisus* isolates from a duodenal biopsy of patient AUS22, while all other lanes were isolated from gum sample of UC patient AUS22. Lanes 1 and 6: *C. concisus* isolates amongst genomospecies A, while other lanes are *C. concisus* isolates belonging to genomospecies B. Lane 7: protein markers. White arrows, brackets and circle indicate distinguishable proteins bands between *C. concisus* isolates. The duodenal isolate has a unique protein profile.

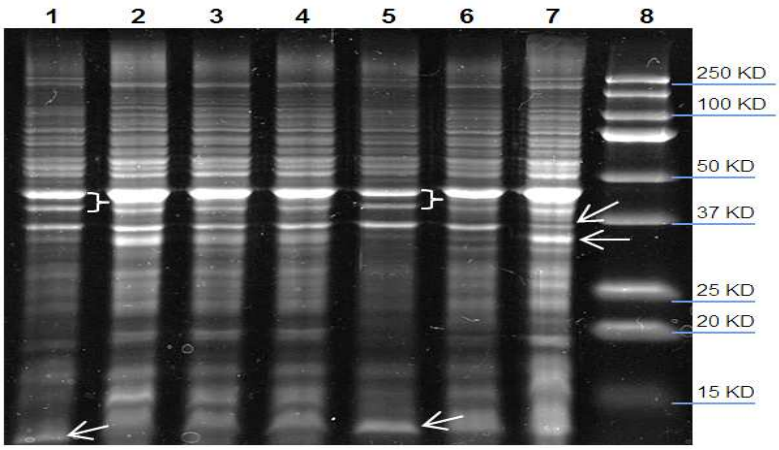


Figure 5.22: SDS-PAGE for whole cell protein profiles of *C. concisus* isolates from gum samples and biopsies collected from patients AUS48 and AUS51. The gel was visualised by Coomassie blue stain. Lanes 1-5: *C. concisus* isolates from AUS48 (AUS48-Ga, AUS48-Gb, AUS48-Gd, AUS48-Ge and AUS48-Ba, respectively). Lanes 6 and 7 are *C. concisus* isolates from AUS51 (AUS51-Ga and AUS51-Bd, respectively). Lane 8: protein marker. Lanes 5 and 7 are *C. concisus* isolates from biopsies of ascending colon (AUS48) and descending colon (AUS51) of UC and CD patients. The other isolates were obtained for gum samples. Here all *C. concisus* isolates are amongst genomospecies B. White arrows and brackets indicate distinguishable proteins bands between *C. concisus* isolates. The protein profile of the isolates obtained from the biopsies are dissimilar to the oral isolates.

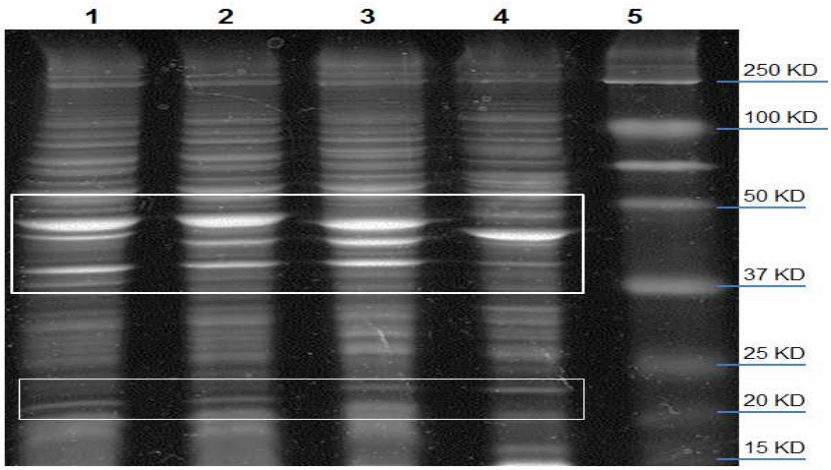


Figure 5.23: SDS-PAGE for whole cell protein profiles of *C. concisus* isolates from a gum and faecal samples collected from control participant AUS47. The gel was visualised by Coomassie blue stain. Lanes 1-4: *C. concisus* isolates (AUS47-Ga, AUS47-Gb, AUS47-Gc and AUS47-Sa, respectively). Lane 5: protein marker. Lanes 1 and 2: *C. concisus* isolates are amongst genomospecies A, while other lanes are *C. concisus* isolates belonging to genomospecies B. Lane 4: a *C. concisus* isolate was obtained from faecal sample of a control participant, while the isolates in the other lanes were obtained from the gum sample. White oblongs indicate distinguishable protein bands between *C. concisus* isolates. The protein profile of the faecal isolate is dissimilar to the oral isolates.

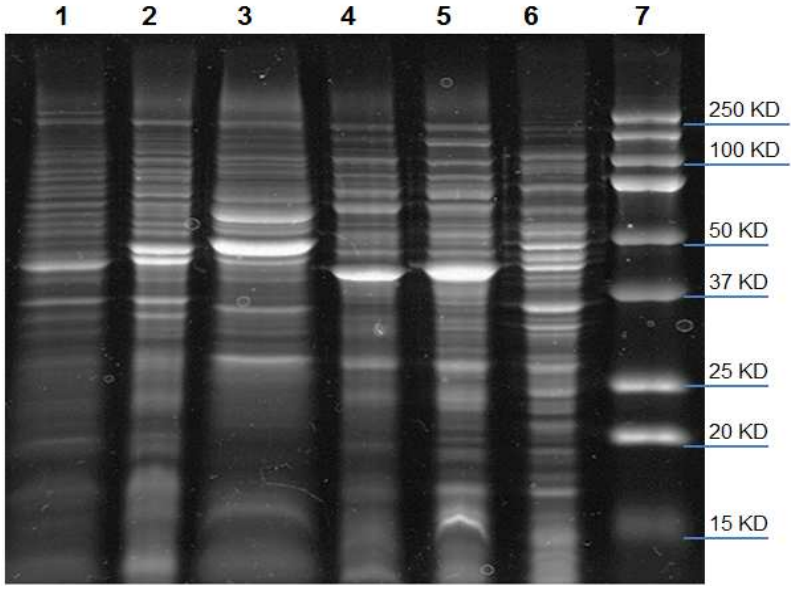


Figure 5.24: SDS-PAGE for whole cell protein profiles of the *Campylobacter* spp. reference strains used in this study. The gel was visualised by Coomassie blue stain. Lanes 1 and 2: *C. concisus* reference strains (ATCC 51561 and 51562). Lane 3-6: *C. mucosalis* ATCC 43264, *C. jejuni* 81116, *C. coli* NCTC 11366 and *E. coli* ATCC 25922, respectively. Lane 7: protein marker. *C. concisus* strain ATCC 51561 was amongst genomospecies B, while ATCC 51562 belonged to genomospecies A.

5.4 Discussion

C. concisus is a bacterium that has generated substantial interest following its detection in colonic biopsies of children with CD. A number of earlier studies reported that *C. concisus* DNA was detected more frequently in intestinal biopsies of newly diagnosed IBD patients than in control participants (Mahendran *et al.* 2011; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009). However, a recent study reported no substantial difference in the detection rate of *C. concisus* between IBD patients and control participants (Hansen *et al.* 2013). One reason for the differences in these results may relate to the different number of the anatomic sites in the intestine where samples were collected in (Hansen *et al.* 2013; Mahendran *et al.* 2011; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009). When the current study was planned in 2010 there was only a single published research article on the prevalence of *C. concisus* DNA extracted from intestinal biopsies of children newly diagnosed with CD. The study reported that the prevalence was higher in CD patients than in control participants (Zhang *et al.* 2009). Therefore, the current study investigated the presence of *C. concisus* by PCR and culture in seven anatomic sites in the intestinal mucosa, as well as faecal and gum samples in adult participants.

In this study, *C. concisus* was isolated from the intestinal biopsies of 8% (3/37) of IBD, while it could not be isolated from control participants. The detection rate of *C. concisus* DNA was 59.5% in intestinal biopsies of IBD patients, while it was 21.4% in control participants, ($P < 0.05$). In addition *C. concisus* was isolated from an intestinal biopsy of the duodenum and ascending colon for the first time. Recently, a single study reported similar finding on the presence of *C. concisus* in four intestinal biopsies (ileum, caecum, descending colon and rectum) of adults with IBD (Mahendran *et al.* 2011). The study also found a significant difference in the detection rate of *C. concisus* DNA between IBD patients (82%) and control participants (48%) (Mahendran *et al.* 2011). However, in another study which was performed

to detect *C. concisus* in intestinal biopsies of paediatric IBD patients, the bacterium was isolated from 3% (3/100) of IBD patients and it failed to reach a significant difference in *C. concisus* detection rate by PCR between IBD patients and control participants (Hansen *et al.* 2013).

In addition, the prevalence of *C. concisus* in biopsies of adults with CD (61.5%) in this study was significantly higher than in control participants (21.4%). This finding is consistent with a study conducted on newly diagnosed children with CD (Zhang *et al.* 2009). However, another study performed on intestinal biopsies of adults with CD, did not detect a significant difference between CD patients and control participants (Mahendran *et al.* 2011).

The differences in the results between this study and the previous studies in the detection rate of *C. concisus* in biopsies of IBD pateints and control participants could be due to the inclusion of different intestinal anatomic sites in different studies as in the current study *C. concisus* was found to be more prevalent in the sigmiod colon and rectum than the other intestinal anatomic sites. Another reason for the different reported detection rates of *C. concisus* could be the additon of different amounts of DNA as a template from the biopsies. Furthermore, nested PCR may be more sensitive than a one-step PCR, which could be important if numbers of *C. concisus* was reduced in the intestinal tract due to the bowel preparation prior endoscopy.

Therefore, as this study used a more comprehensive sampling strategy than previous studies, the findings have for the first time expanded the knowledge of *C. concisus* prevalence in several anatomic sites in the gastrointestinal tract of adults with IBD. These results showed that *C. concisus* can be detected in any of these sites including duodenal and stomach biopsies.

In the current study, *C. concisus* was isolated by culture from gum samples of 86% (32/37) of IBD patients and of 64% (9/14) of control participants. The detection rate of *C. concisus* by

PCR was 100% in IBD patients and 97% in control participants. However, the differences in detection rate by both culture method and PCR were not statistically significant between IBD patients and control participants. A similar finding was reported in a previous study conducted on saliva of children and adults with IBD as there was no statistical significance in the prevalence of *C. concisus* between IBD patients and control participants (Zhang *et al.* 2010). The preparation for the endoscopy procedure used in this study could have reduced the numbers of *C. concisus* in the oral cavity and may have led to a slight reduction in the bacterial isolation rate, compared with the PCR detection rate.

The isolation rate of *C. concisus* from faecal samples in the present study was 5.6% (2/36) in IBD patients and 8.3% (1/12) in control participants, which was not statistically significant. The prevalence of *C. concisus* DNA in faecal samples of IBD patients was higher than in control participants, but the difference did not reach statistical significance. However, a previous study recruited faecal samples from children with newly diagnosed CD and found that the prevalence of *C. concisus* DNA in faecal specimens of CD patients (65%) was significantly higher than in control participants (33%) (Man *et al.* 2010b). The variation in the detection rate between this study and the past study may be due to the differences in the age groups of the participants.

The findings of this study for the first time revealed that the prevalence of genomospecies B was significantly higher in intestinal biopsies and faecal samples of IBD patients than in control participants (P <0.05) when a newly developed nested PCR technique to amplify *cjaC* was used. In addition, the prevalence of genomospecies B in *C. concisus* isolates from gum samples of IBD patients was significantly higher than genomospecies A, while in control participants, genomospecies A was more prevalent than genomospecies B. Similar results were obtained when the new nested PCR was applied to detect genomospecies B. These results suggest that a specific genomospecies may be associated with IBD. As the

whole genome sequence of a genomospecies A strain (ATCC 51562) has recently become available in the NCBI data-base site (Deshpande *et al.* 2013), the prevalence of this genomospecies can be investigated by developing a nested PCR targeting specific genes of interest to detect genomospecies A and support the findings of this study.

The *zot* gene was suggested to play a role during bacterial infections by mimicking intercellular tight cell junctions (Uzzau *et al.* 2000) and in *Campylobacter* spp., only *C. concisus* and *C. ureolyticus* have been reported to possess *zot* in their genomes (Bullman *et al.* 2013; Kaakoush *et al.* 2010). The current study found that 8% (3/37) of isolates from the oral cavity of IBD patients possessing *zot* compared with 14% (2/14) of controls. This difference did not reach statistical significance. When Mahendran *et al.* (2013) investigated the prevalence of *zot* within *C. concisus* oral isolates obtained from IBD patients and control participants, they also could not show statistical significance.

A new nested PCR developed in the present study to detect *zot* in the extracted DNA from clinical samples, also found no statistical significance in the prevalence of *zot* in clinical samples between IBD patients and control participants ($P > 0.05$). Based on the sequence of *C. concisus* 13826, polymorphic forms of *zot* leading to a substitution of valine to leucine at position 270 were reported in *C. concisus* strains and linked to patients with active IBD (Mahendran *et al.* 2013). However, this study found that this kind of substitution in *C. concisus* isolates obtained from control participants.

In this study, *C. concisus* isolates obtained from faecal samples of IBD patients demonstrated identical WCLP patterns to oral isolates obtained from the same patients. However, intestinal isolates obtained from biopsies of the same patient showed dissimilar WCLP patterns to the oral isolates. These differences possibly suggest that isolates obtained from biopsies express factors which may facilitate the colonisation of intestinal epithelial cells; however, more

work is required to identify the proteins specific to IBD isolates and their function, and to compare DNA profiles of isolates from different sites of the same patient.

As part of the plan for this study, serum samples were also collected from participants when the biopsies, faecal samples and gum samples were taken. However due to time limitation no experimental analysis was conducted; therefore, further studies are required to perform protein immunoreactive studies for *C. concisus* strains isolated from different locations within the same patient. The hypothesis to be investigated was that only patients who have *C. concisus* in biopsies may have immunoreactivity against *C. concisus*. In addition, interleukins and IgM studies are suggested for the patients who had *C. concisus* in the biopsies and compared with results to the patients who do not have the bacterium in their biopsies.

In summary, *C. concisus* was detected in intestinal biopsies of IBD patients including from new intestinal anatomic sites which have not been investigated by previous studies that recruited adults with IBD and control participants. These anatomic intestinal sties were ascending colon, transverse colon, sigmoid colon, duodenum and stomach. The study revealed that the prevalence of *C. concisus* in intestinal biopsies of IBD patients was significantly higher than in the control participants. In addition it was the first study to look at the prevalence of different genomospecies in *C. concisus*, using DNA collected from clinical samples (biopsies, faeces and gum) collected from IBD patients.

C. concisus was isolated for the first time from biopsies collected from duodenum and ascending colon of IBD patients. In addition, this was the first study to reveal that the prevalence of genomospecies B strains in clinical samples was significantly higher in IBD patients than in control participants while genomospecies A strains were more predominant in control participants than in IBD patients suggesting that genomospecies B could be more associated with IBD than genomospecies A. The isolation rate of *C. concisus* from biopsies and faeces of IBD patients and control participants was significantly lower compared to the

oral cavity. *C. concisus* isolates even from same genomospecies obtained from biopsies showed unique WCLP patterns when compared with oral isolates from the same patient unlike faecal isolates whose profiles were identical with the oral isolates, possibly suggesting that the isolates obtained from biopsies could have several proteins that facilitate their survival in intestinal cells. The prevalence of *C. concisus* isolates possessing *zot* was extremely low in IBD patients and control participants indicating that the gene might be unimportant for bacterial colonisation in the intestinal tract or as has been suggested it may have been acquired from other microorganisms (Zhang *et al.* 2014). It is still unclear whether *C. concisus* has any involvements in IBD or whether patients with IBD are more susceptible to being colonised by the bacterium than control participants.

These findings suggest that further studies are required to confirm the variations of WCLP profiles between isolates obtained from biopsies and the oral cavity in the same IBD patient. Further genetic analysis also should be considered using *C. concisus* isolates from genomospecies A and B. To my knowledge, this is first study that has isolated *C. concisus* from the intestine and the oral cavity of the same IBD patient at the same time. These outcomes suggested that further investigations are required to detect the prevalence of *C. concisus* genetic groups direct from the extracted DNA of clinical samples as its isolation is very challenging due to being an extremely fastidious bacterium. In order to achieve this completely, whole genome sequencing for other genomospecies A strains is required.

Chapter 6 : General discussion

In the past decade, several studies reported an emergence of *C. concisus* as a possible pathogen in the gastrointestinal tract (Maher *et al.* 2003; On 2001; Park 2002). *C. concisus* has been detected in patients with gum infection (Kamma *et al.* 1994; Kamma *et al.* 2001; Tanner *et al.* 1981) and in the faeces of gastroenteritis patients (Istivan *et al.* 2004; Lindblom *et al.* 1995; Vandamme *et al.* 1989). Since 2009, a few studies have reported that the detection of *C. concisus* DNA was significantly higher in intestinal biopsies from patients with IBD than from healthy control (Mahendran *et al.* 2011; Zhang *et al.* 2009), while other studies could not detect a significant difference (Hansen *et al.* 2013; Hansen *et al.* 2011). These findings do not make it possible to draw a conclusion on the role of *C. concisus* in enteric infections at present.

C. concisus has been grouped into two genomospecies (A & B) by amplification of the 23S rDNA region (Aabenhus *et al.* 2005a; Istivan 2005; Istivan *et al.* 2004) and into 3-4 groups by AFLP (Aabenhus *et al.* 2005a; Kalischuk *et al.* 2011). Genomospecies A and B were reported to correspond with group I and II that were based on AFLP analysis (Aabenhus *et al.* 2005a).

Due to the genetic diversity of this species and due to different typing methods used to discriminate *C. concisus* strains, the DGGE technique can be used to investigate and differentiate *C. concisus* in mixed bacterial communities such as the intestine and the oral cavity. In the current study, *C. concisus* isolates were typed into two new groups (I and II) by the DGGE technique based on the analysis of the 16S rDNA gene. Genomospecies A isolates were assigned into two DGGE groups; however, genomospecies B isolates were all assigned into one group. Using different genes to type *C. concisus* may result in generating diverse new groups.

The *C. concisus* genome has been reported to possess several putative virulence genes; *cjaC, cjaA, dnaJ* and *zot* (Kaakoush *et al.* 2010). In *C. jejuni*, *cjaC* and *cjaA* encode secreted proteins that are immunodominant (Laniewski *et al.* 2014; Muller *et al.* 2005; Wyszynska *et al.* 2004). These genes are predicted to be putatively responsible for histidine and cysteine up-take in campylobacters (Muller *et al.* 2005; Wyszynska *et al.* 2004; Wyszynska *et al.* 2007). The *dnaJ* gene encodes the chaperone protein DnaJ, which has been reported as a virulence-associated gene in *C. jejuni* (Bui *et al*. 2012; Li *et al*. 2008). The *zot* gene encodes the zonula occludens toxin, and has been detected in the genome of several *C. concisus* strains (Kaakoush *et al.* 2010; Kalischuk *et al.* 2011). This gene is known to increase the permeability of intestinal cells in the enteric infection caused by *V. cholera* (Di Pierro *et al.* 2001; Fasano *et al.* 1995).

In this study, results of PCR amplification and bioinformatics analysis revealed that the majority of *C. concisus* isolates carry *cjaC*, *cjaA* and *dnaJ*, while *zot* was possessed by only a few isolates. The DNA and amino acid sequences published for *cjaC, cjaA* and *dnaJ* were found to be substantially different between genomospecies A and B, which might explain the failure of PCR to amplify the genes from some *C. concisus* isolates. Based on alignment of a DNA sequence obtained from *C. concisus* strain RCH3 (genomospecies A) in this study to *C. concisus* strain 13826 (genomospecies B), the location of *cjaC* is in different parts of the genome in genomospecies A and B. This study found that the nucleotide sequence of the region between *cjaC* and the shikimate 5-dehydrogenase genes in genomospecies A is unique; therefore, it can be targeted to detect genomospecies A. Using PCR with the panel of primer sets based on *C. concisus* 13826 to amplify the DNA region between *cjaC* and the hypothetical protein gene (CCC13826_0963), it showed that genomospecies B isolates are heterogonous. Moreover, *cjaA, dnaJ* and *zot* could not be amplified from all *C. concisus* isolates with the primer sets designed based on the *C. concisus* 13826 sequence. This inability

to amplify these genes from all isolates was likely to be due to sequence variations, since alignment of published sequences of *C. concisus* strains (once these became available) showed the sequences of these genes to be heterogeneous.

Neighbour-joining phylogenetic analysis for *cjaC*, *cjaA* and *dnaJ* using DNA and corresponding amino acid sequences of published *C. concisus* strains confirmed that the nucleotide sequences of these genes differ between genomospecies A and B, as analysis based on all of these genes showed that each genomospecies had its own clade. Phylogenetic analysis based on *zot* was limited since this gene could only be detected in three out of the eight whole sequenced *C. concisus* strains (13826, UNSW3 and UNSWCS), all of which were from genomospecies B. The group assignment obtained from *zot* sequence analysis by neighbour-joining phylogenetic analysis was different from that according to 23S rDNA, *cjaC*, *dnaJ* and *cjaA*. In addition, isolates possessing *zot*, were assigned into the same group by DGGE analysis based on the PCR product of primer set I.

The present study found that two oral *C. concisus* isolates obtained from healthy volunteers were invasive to INT407 cells (invasion index values >1). However, Ismail *et al.* (2012) detected *C. concisus* isolates capable of invading Caco-2 cells in the oral cavity of IBD patients, but not from the oral cavity of healthy controls. These differences could be explained if the invasion of *C. concisus* differs based on the used cell line. In this study, *C. concisus* isolates that possessed *zot* were non-invasive (invasive index value <1). More studies are required to determine whether *zot* is involved in bacterial interaction with intestinal cells *in vitro*.

In the present study semi-quantitative RT-PCR assays were developed to investigate the gene expression of putative virulence genes (*cjaC*, *cjaA*, *dnaJ* and *zot*). The assays successfully detected and evaluated the expression of these genes with the exception of *zot*, which was not expressed under the experimental condition. The expression of *cjaC* and *cjaA* was weakly

induced by alteration of the growth medium. The expression of *dnaJ* was found to be significantly reduced when *C. concisus* cells were maintained in DMEM (a tissue culture medium) compared to when these bacterial cells were maintained with INT407 cells growing in DMEM. These results indicate different expression levels under different growth conditions. It is likely that similar differences in expression of virulence genes also occurs *in vivo* in the human host, but more work is required to confirm this.

A few studies have reported a significantly higher prevalence of *C. concisus* DNA in intestinal biopsies of children newly diagnosed with IBD than in healthy controls (Mahendran *et al.* 2011; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009). However, more recent study could not show a significant difference in the detection rate of *C. concisus* in colonic biopsies between IBD patients and healthy controls (Hansen *et al.* 2013). The earlier studies did not examine different samples of the gastrointestinal tract and the oral cavity of each IBD patient at the same time. Therefore, in the present study samples were collected from intestinal biopsies, faeces and gum swabs from patients with IBD at the Austin Hospital and transported to RMIT University for laboratory investigations. Prior to sample collection, the effect of transportation temperature on the survival rate of *C. concisus* was investigated as this has not been previously reported. A temperature of 4°C was used as a model to investigate the effect of transport conditions on *C. concisus* cell survival. There was no significant reduction in *C. concisus* cell numbers in suspensions stored at 4°C for 7 h, which was the required time from sample collection through transportation to processing by culture. The rates of retrieval of genomospecies A from bacterial storage at 4°C were significantly higher after 2 days at 4°C than for genomospecies B. This suggested that a delay in cultivation of clinical samples beyond this point (after 2 days at 4°C) may not show the accurate prevalence of genomospecies A and B as genomospecies B had lower survival rates than genomospecies A.

Previous studies collected biopsies from only a few intestinal sites. For example, Mahendran *et al.* (2011) determined the prevalence of *C. concisus* DNA in four anatomic sites (ileum, caecum, descending colon and rectum), while other studies analysed *C. concisus* DNA only in a colonic biopsy collected from a single site of each IBD patient (Hansen *et al.* 2013; Mukhopadhya *et al.* 2011; Zhang *et al.* 2009). In contrast, in the present study, *C. concisus* prevalence was investigated in biopsies from additional anatomical sites in intestinal mucosa (the terminal ileum, ascending colon, transverse colon and sigmoid colon) using PCR and culture. In some participants, stomach and duodenal biopsies were also collected. Furthermore, new PCR techniques were developed to detect *C. concisus* genomospecies B and *zot* directly from the clinical samples.

The prevalence of *C. concisus* DNA in intestinal biopsies of patients with IBD (59.5%) was significantly higher than in control participants (21.4%), ($P < 0.05$). Similar results was previously shown by Mahendran *et al.* (2011). Moreover, it was the first study to show a statistically significant prevalence of *C. concisus* DNA in intestinal biopsies of adults with CD (61.5%) when compared to healthy controls, while Mahendran *et al.* (2011) could not detect a significant difference between CD patients and controls. These differences could be due to using more intestinal anatomic sites from each patient in the current study. In the oral cavity, this study and a previous study found no significant differences in the prevalence of *C. concisus* between of patients IBD and healthy controls using PCR and culture (Zhang *et al.* 2010). In faecal samples collected in this study, there was no significant difference in the prevalence of *C. concisus* between IBD patients and control participants, while Man *et al.* (2010b) reported a significant difference between children with CD and control participants; these dissimilar results may be due to the different age groups of participants.

This was the first study to focus on the prevalence of specific genomospecies of *C. concisus* in intestinal biopsies, faeces and gum samples of patients with IBD. Using PCR and culture

to detect *C. concisus* in the oral cavity, the prevalence of genomospecies B was significantly higher in IBD patients than in control participants, while genomospecies A isolates were more prevalent in control participants than in IBD patients. Similar results were found when PCR was applied to detect genomospecies B in intestinal biopsies and faeces, suggesting that genomospecies B could be more associated with IBD than genomospecies A as genomospecies B has a significant prevalence in all sites of these patients.

Using PCR, there was no significant difference in the rate of detection of *zot* in intestinal biopsies, faeces or the oral cavity between IBD patients and control participants. Similar results were found when *zot* was investigated in the oral isolates of IBD patients and controls. Mahendran *et al.* (2013) also reported that there was no statistically significant difference in the prevalence of *zot* in *C. concisus* isolates obtained from IBD patients and control participants. Together, these findings suggest that *zot* might not be associated with IBD.

As *C. concisus* isolates are genetically heterogonous, the phenotypic similarity between *C. concisus* isolates obtained from the oral cavity and the gastrointestinal tract of the same patient collected at the same time was investigated by whole cell lysate protein profiling using SDS-PAGE. *C. concisus* isolates obtained from biopsies showed unique protein profiles compared to the oral isolates from the same IBD patient. However, the protein profiles of faecal isolates were identical to those of oral isolates obtained from IBD patients. These results suggest that isolates from intestinal biopsies could be different from those colonising the oral cavity.

The conclusive results obtained from IBD indicated that the prevalence of *C. concisus* in intestinal biopsies of IBD patients was significantly higher than in control participants. In addition, genomospecies B prevalence in biopsies, faeces and oral cavity of IBD patients was significantly higher than in control participants. Based on protein profile analysis, isolates

obtained from the biopsies of IBD patients were different from *C. concisus* isolates colonising the oral cavity of the same patient.

In summary, the DGGE technique separated *C. concisus* isolates into two new distinct groups. Additionally, the putative virulence genes (*cjaC*, *cjaA* and *dnaJ*) may be possessed by the majority of *C. concisus* isolates, but they exhibit nucleotide sequence variations. However, *zot* could be only amplified from few isolates. The location of *cjaC* in the genome of genomospecies A strains was different from its location in the genome of genomospecies B strains. Two oral *C. concisus* isolates obtained from healthy individuals were identified as invasive, which is the first time to be reported. In addition, semi-quantitative SYBR green assays were developed to evaluate the expression of putative virulence genes in *C. concisus* isolates grown on synthetic media and in isolates maintained in tissue culture medium with and without INT407 cells. The expression of *cjaC* and *cjaA* was slightly enhanced when the bacterial cells were grown in an unenriched medium. However, *dnaJ* expression was significantly reduced in bacterial cells maintained in the tissue culture medium only than in bacterial cells maintained in INT407 cells. New nested PCR techniques were developed to detect genomospecies B isolates and *zot* clinical samples. The prevalence of *C. concisus* was significantly higher in biopsies of IBD patients than in biopsies of healthy controls. *C. concisus* was isolated from duodenal and ascending colonic biopsies, for the first time in this study. The prevalence of genomospecies B isolates was significantly higher in IBD patients (35.1%) than in control participants (7.1%). However, using PCR and culture, there was no significant difference in the prevalence of *zot* between IBD patients and control participants.

Future studies

It is proposed that the genetic diversity of *C. concisus* may indicate differences in the degree of virulence; however, further studies are required to evaluate differences in virulence between genetically diverse isolates and between genomospecies A and B. Additional whole

genome sequencing for genomospecies A strains is required to facilitate the detection and investigation of putative virulence genes of *C. concisus*. Proteomic studies on *cjaC, cjaA, zot* and *dnaJ* gene products should investigate whether there is any difference in the products that could affect bacterial virulence. Targeting 23S rDNA by nested PCR, several molecular techniques such as DGGE can be used directly to group *C. concisus* DNA in clinical samples. As this study established a foundation for identifying relative gene expression for *C. concisus*, further studies should be undertaken in order to determine the expression levels of the other putative virulence genes. Investigations are required to confirm protein profile differences between isolates obtained from biopsies and oral isolates of the same patients. More investigations are needed to study the pathogenic potential of *C. concisus* isolates obtained from intestinal biopsies and faecal samples of IBD patients compared to isolates from the oral cavity of the same patient. Further studies may need to be done using animal models with *C. concisus* isolates to investigate and confirm the pathogenic role of *C. concisus*, in particular those isolated from the intestinal tract and oral cavity of the same IBD patient. Generation of targeted mutation for proposed virulence genes in *in vitro* and animal models would also be needed to confirm their role, if any in intestinal disease. Additional information would be gained by investigating the host humoral immune response to the *C. concisus* in IBD patients compared with control participants. Furthermore, the immunoreactivity of patient sera against proteins from *C. concisus* isolates obtained from different locations (intestinal biopsies, faeces and the oral cavity) against the serum of the same patient would provide an insight into whether the immune response is direct against all isolates or only isolates obtained from intestinal biopsies.

References

Aabenhus, R., On, SL., Siemer, BL., Permin, H & Andersen, LP (2005a), 'Delineation of *Campylobacter concisus* genomospecies by amplified fragment length polymorphism analysis and correlation of results with clinical data', *J Clin Microbiol*, vol. 43, no. 10, pp. 5091-6.

Aabenhus, R., Permin, H & Andersen, LP (2005b), 'Characterization and subgrouping of *Campylobacter concisus* strains using protein profiles, conventional biochemical testing and antibiotic susceptibility', *Eur J Gastroenterol Hepatol*, vol. 17, no. 10, pp. 1019-24.

Aabenhus, R., Permin, H., On, SL & Andersen, LP (2002), 'Prevalence of *Campylobacter concisus* in diarrhoea of immunocompromised patients', *Scand J Infect Dis*, vol. 34, no. 4, pp. 248-52.

Allos, BM (2001), '*Campylobacter jejuni* infections: update on emerging issues and trends', *Clin Infect Dis*, vol. 32, no. 8, pp. 1201-6.

Altekruse, SF., Stern, NJ., Fields, PI & Swerdlow, DL (1999), '*Campylobacter jejuni*--an emerging foodborne pathogen', *Emerg Infect Dis*, vol. 5, no. 1, pp. 28-35.

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., De Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., Xenarios, I & Stockinger, H (2012), 'ExPASy: SIB bioinformatics resource portal', *Nucleic Acids Res*, vol. 40, no. Web Server issue, pp. W597-603.

Bastyns, K., Chapelle, S., Vandamme, P., Goossens, H & De Wachter, R (1995), 'Specific detection of *Campylobacter concisus* by PCR amplification of 23S rDNA areas', *Mol Cell Probes*, vol. 9, no. 4, pp. 247-50.

Bereswill, S & Kist, M (2002), 'Molecular microbiology and pathogenesis of *Helicobacter* and *Campylobacter* updated: a meeting report of the 11th conference on *Campylobacter, Helicobacter* and related organisms', *Mol Microbiol*, vol. 45, no. 1, pp. 255-62.

Bjrkholm, B., Lundin, A., Sillen, A., Guillemin, K., Salama, N., Rubio, C., Gordon, JI., Falk, P & Engstrand, L (2001), 'Comparison of genetic divergence and fitness between two subclones of *Helicobacter pylori*', *Infect. Immun*, vol. 69, pp. 7832-8.

Blackett, KL., Siddhi, SS., Cleary, S., Steed, H., Miller, MH., Macfarlane, S., Macfarlane, GT & Dillon, JF (2013), 'Oesophageal bacterial biofilm changes in gastro-oesophageal reflux disease, Barrett's and oesophageal carcinoma: association or causality?', *Aliment Pharmacol Ther*, vol. 37, no. 11, pp. 1084-92.

Blaser, MJ (1998), '*Campylobacter fetus*--emerging infection and model system for bacterial pathogenesis at mucosal surfaces', *Clin Infect Dis*, vol. 27, no. 2, pp. 256-8.

Blaser, MJ., Taylor, DN & Feldman, RA (1983), 'Epidemiology of *Campylobacter jejuni* infections', *Epidemiol Rev*, vol. 5, pp. 157-76.

Boehm, M., Lind, J., Backert, S & Tegtmeyer, N (2015), '*Campylobacter jejuni* serine protease HtrA plays an important role in heat tolerance, oxygen resistance, host cell adhesion, invasion, and transmigration', *European Journal of Microbiology and Immunology*, vol. 5, no. 1, pp. 68-80.

Bohr, UR., Glasbrenner, B., Primus, A., Zagoura, A., Wex, T & Malfertheiner, P (2004), 'Identification of enterohepatic *Helicobacter* species in patients suffering from inflammatory bowel disease', *J Clin Microbiol*, vol. 42, no. 6, pp. 2766-8.

Buelow, DR., Christensen, JE., Neal-Mckinney, JM & Konkel, ME (2011), '*Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI', *Molecular microbiology*, vol. 80, no. 5, pp. 1296-312.

Bui, XT., Qvortrup, K., Wolff, A., Bang, DD & Creuzenet, C (2012), 'Effect of environmental stress factors on the uptake and survival of *Campylobacter jejuni* in Acanthamoeba castellanii', *BMC Microbiol*, vol. 12, no. 1, p. 232.

Bullman, S., Lucid, A., Corcoran, D., Sleator, RD & Lucey, B (2013), 'Genomic investigation into strain heterogeneity and pathogenic potential of the emerging gastrointestinal pathogen *Campylobacter ureolyticus*', *PLoS One*, vol. 8, no. 8, p. e71515.

Burgos-Portugal, JA., Mitchell, HM., Castano-Rodriguez, N & Kaakoush, NO (2014), 'The role of autophagy in the intracellular survival of *Campylobacter concisus*', *FEBS Open Bio*, vol. 4, pp. 301-9.

Chaban, B., Musil, KM., Himsworth, CG & Hill, JE (2009), 'Development of cpn60-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples', *Appl Environ Microbiol*, vol. 75, no. 10, pp. 3055-61.

Chaban, B., Ngeleka, M & Hill, JE (2010), 'Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals', *BMC Microbiol*, vol. 10, p. 73.

Chambers, DA., Imrey, PB., Cohen, RL., Crawford, JM., Alves, ME & Mcswiggin, TA (1991), 'A longitudinal study of aspartate aminotransferase in human gingival crevicular fluid', *J Periodontal Res*, vol. 26, no. 2, pp. 65-74.

Chandrashekhar, K., Gangaiah, D., Pina-Mimbela, R., Kassem, II., Jeon, BH & Rajashekara, G (2015), 'Transducer like proteins of *Campylobacter jejuni* 81-176: role in chemotaxis and colonization of the chicken gastrointestinal tract', *Front Cell Infect Microbiol*, vol. 5, p. 46.

Chang, J (2002), 'A study of the invasion characteristics of *Campylobacter jejuni*', PhD thesis RMIT University, Australia.

Chansiripornchai, N & Sasipreeyajan, J (2009), 'PCR detection of four virulence-associated genes of *Campylobacter jejuni* isolates from Thai broilers and their abilities of adhesion to and invasion of INT-407 cells', *J Vet Med Sci*, vol. 71, no. 6, pp. 839-44.

Chua, K., Gürtler, V., Montgomery, J., Fraenkel, M., Mayall, BC & Grayson, ML (2007), '*Campylobacter insulaenigrae* causing septicaemia and enteritis', *J Med Microbiol*, vol. 56, no. 11, pp. 1565-7.

Clark, JD., Oakes, RD., Redhead, K., Crouch, CF., Francis, MJ., Tomley, FM & Blake, DP (2012), '*Eimeria* species parasites as novel vaccine delivery vectors: anti-*Campylobacter jejuni* protective immunity induced by *Eimeria tenella*-delivered CjaA', *Vaccine*, vol. 30, no. 16, pp. 2683-8.

Collado, L., Gutierrez, M., Gonzalez, M & Fernandez, H (2013), 'Assessment of the prevalence and diversity of emergent campylobacteria in human stool samples using a combination of traditional and molecular methods', *Diagn Microbiol Infect Dis*, vol. 75, no. 4, pp. 434-6.

Colletti, T (2004), 'IBD--recognition, diagnosis, therapeutics', *JAAPA*, vol. 17, no. 5, pp. 16-8, 21-4.

Cornelius, AJ., Chambers, S., Aitken, J., Brandt, SM., Horn, B & On, SL (2012), 'Epsilonproteo-bacteria in Humans, New Zealand'.

Cox, CJ., Kempsell, KE & Gaston, JS (2003), 'Investigation of infectious agents associated with arthritis by reverse transcription PCR of bacterial rRNA', *Arthritis Res Ther*, vol. 5, no. 1, pp. R1-8.

De Vries, JJ., Arents, NL & Manson, WL (2008), '*Campylobacter* species isolated from extra-oro-intestinal abscesses: a report of four cases and literature review', *Eur J Clin Microbiol Infect Dis*, vol. 27, no. 11, pp. 1119-23.

Debruyne, L., On, SL., De Brandt, E & Vandamme, P (2009), 'Novel *Campylobacter lari*-like bacteria from humans and molluscs: description of *Campylobacter peloridis* sp. nov., *Campylobacter lari* subsp. concheus subsp. nov. and *Campylobacter lari* subsp. lari subsp. nov', *Int J Syst Evol Microbiol*, vol. 59, no. Pt 5, pp. 1126-32.

Deshpande, NP., Kaakoush, NO., Mitchell, H., Janitz, K., Raftery, MJ., Li, SS & Wilkins, MR (2011), 'Sequencing and validation of the genome of a *Campylobacter concisus* reveals intra-species diversity', *PLoS One*, vol. 6, no. 7, p. e22170.

Deshpande, NP., Kaakoush, NO., Wilkins, MR & Mitchell, HM (2013), 'Comparative genomics of *Campylobacter concisus* isolates reveals genetic diversity and provides insights into disease association', *BMC Genomics*, vol. 14, p. 585.

Di Pierro, M., Lu, R., Uzzau, S., Wang, W., Margaretten, K., Pazzani, C., Maimone, F & Fasano, A (2001), 'Zonula occludens toxin structure-function analysis identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain', *Journal of Biological Chemistry*, vol. 276, no. 22, pp. 19160-5.

Donlan, RM (2002), 'Biofilms: microbial life on surfaces', *Emerg Infect Dis*, vol. 8, no. 9, pp. 881-90.

Ebersole, JL., Taubman, MA., Smith, DJ & Haffajee, AD (1985), 'Effect of subgingival scaling on systemic antibody responses to oral microorganisms', *Infect Immun*, vol. 48, no. 2, pp. 534-9.

Elsinghorst, EA (1994), 'Measurement of invasion by gentamicin resistance', *Methods Enzymol*, vol. 236, pp. 405-20.

Engberg, J., Bang, DD., Aabenhus, R., Aarestrup, FM., Fussing, V & Gerner-Smidt, P (2005), '*Campylobacter concisus*: an evaluation of certain phenotypic and genotypic characteristics', *Clin Microbiol Infect*, vol. 11, no. 4, pp. 288-95.

Engberg, J., On, SL., Harrington, CS & Gerner-Smidt, P (2000), 'Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for Campylobacters', *J Clin Microbiol*, vol. 38, no. 1, pp. 286-91.

Eucker, TP & Konkel, ME (2012), 'The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal *Campylobacter jejuni* invasion of host cells by stimulating membrane ruffling', *Cellular microbiology*, vol. 14, no. 2, pp. 226-38.

Fasano, A., Fiorentini, C., Donelli, G., Uzzau, S., Kaper, JB., Margaretten, K., Ding, X., Guandalini, S., Comstock, L & Goldblum, SE (1995), 'Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro', *J Clin Invest*, vol. 96, no. 2, pp. 710-20.

Fath, MJ & Kolter, R (1993), 'ABC transporters: bacterial exporters', *Microbiol Rev*, vol. 57, no. 4, pp. 995-1017.

Faul, F., Erdfelder, E., Lang, A-G & Buchner, A (2007), 'G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences', *Behavior research methods*, vol. 39, no. 2, pp. 175-91.

Fearnley, C., Manning, G., Bagnall, M., Javed, MA., Wassenaar, TM & Newell, DG (2008), 'Identification of hyperinvasive *Campylobacter jejuni* strains isolated from poultry and human clinical sources', *J Med Microbiol*, vol. 57, no. 5, pp. 570-80.

Felsenstein, J (1989), 'phylip: Phylogeny Inference Package (version 3.2)', *Cladistics*, no. 5, pp. 164 - 6.

Fiocchi, C (1998), 'Inflammatory bowel disease: etiology and pathogenesis', *Gastroenterology*, vol. 115, no. 1, pp. 182-205.

Fischer, SG & Lerman, LS (1983), 'DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory', *Proc Natl Acad Sci U S A*, vol. 80, no. 6, pp. 1579-83.

Fitzgerald, C., Whichard, J & Nachamkin, I (2008), Dignosis and antimicrobial susceptibility of *Campylobacter* species, ASM Press, Washington, in I Nachamkin, CM Szymanski & MJ Blaser (eds), *Campylobacter*, 3rd edn.

Fouts, E., Mongodin, F., Puiu, D., Sebastian, Y., Miller, G., Mandrell, E., On, S & Nelson, E (2007), 'Genome sequence of *Campylobacter concisus* 13826 isolated from human feces', *EMBL/GenBank/DDBJ databases*.

Friedman, CR., Hoekstra, RM., Samuel, M., Marcus, R., Bender, J., Shiferaw, B., Reddy, S., Ahuja, SD., Helfrick, DL., Hardnett, F., Carter, M., Anderson, B., Tauxe, RV & Emerging Infections Program Foodnet Working, G (2004), 'Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites', *Clin Infect Dis*, vol. 38 Suppl 3, pp. S285-96.

Fuss, IJ., Neurath, M., Boirivant, M., Klein, JS., De La Motte, C., Strong, SA., Fiocchi, C & Strober, W (1996), 'Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5', *J Immunol*, vol. 157, no. 3, pp. 1261-70.

Garvis, SG., Puzon, GJ & Konkel, ME (1996), 'Molecular characterization of a *Campylobacter jejuni* 29-kilodalton periplasmic binding protein', *Infect Immun*, vol. 64, no. 9, pp. 3537-43.

Grönroos, E., Thodeti, CK & Sjölander, A (1998), 'Leukotriene D 4 induces a rapid increase in cAMP in the human epithelial cell line, Int 407: a potential role for this signal in the regulation of calcium influx through the plasma membrane', *Cell calcium*, vol. 24, no. 1, pp. 9-16.

Gunther, NW & Chen, CY (2009), 'The biofilm forming potential of bacterial species in the genus *Campylobacter*', *Food Microbiol*, vol. 26, no. 1, pp. 44-51.

Guslandi, M., Zhang, L., Man, SM., Day, AS., Leach, ST., Lemberg, DA., Dutt, S., Stormon, M., Otley, A., O'loughlin, EV., Magoffin, A., Ng, PH & Mitchell, H (2009), '*Campylobacter concisus*: a new character in the Crohn's disease story?', *J Clin Microbiol*, vol. 47, no. 5, pp. 1614-5.

Haffajee, AD., Socransky, SS., Ebersole, JL & Smith, DJ (1984), 'Clinical, microbiological and immunological features associated with the treatment of active periodontosis lesions', *J Clin Periodontol*, vol. 11, no. 9, pp. 600-18.

Haiko, J & Westerlund-Wikström, B (2013), 'The role of the bacterial flagellum in adhesion and virulence', *Biology*, vol. 2, no. 4, pp. 1242-67.

Hamidian, M., Sanaei, M., Bolfion, M., Dabiri, H., Zali, MR & Walther-Rasmussen, J (2011), 'Prevalence of putative virulence markers in *Campylobacter jejuni* and *Campylobacter coli* isolated from hospitalized children, raw chicken, and raw beef in Tehran, Iran', *Can J Microbiol*, vol. 57, no. 2, pp. 143-8.

Hanauer, SB (2006), 'Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities', *Inflamm Bowel Dis*, vol. 12, no. 5, pp. S3-S9.

Hansen, R., Berry, SH., Mukhopadhya, I., Thomson, JM., Saunders, KA., Nicholl, CE., Bisset, WM., Loganathan, S., Mahdi, G., Kastner-Cole, D., Barclay, AR., Bishop, J., Flynn, DM., Mcgrogan, P., Russell, RK., El-Omar, EM & Hold, GL (2013), 'The microaerophilic microbiota of de-novo paediatric inflammatory bowel disease: the BISCUIT study', *PLoS One*, vol. 8, no. 3, p. e58825.

Hansen, R., Mukhopadhya, I., Russell, RK., Bisset, WM., Berry, SH., Thomson, JM., El-Omar, EM & Hold, GL (2011), 'The role of microaerophilic colonic mucosal bacteria in de novo paediatric inflammatory bowel disease', *Gut 60*, vol. (Suppl. 1), p. A147.

Henle, G & Deinherdt, F (1957), 'The establishment of strains of human cells in tissue culture', *J. Immunol*, vol. 79, pp. 54-9.

Hess, DL., Pettersson, AM., Rijnsburger, MC., Herbrink, P., Van Den Berg, HP & Ang, CW (2012), 'Gastroenteritis caused by *Campylobacter concisus*', *J Med Microbiol*, vol. 61, no. Pt 5, pp. 746-9.

Higgins, CF (1995), 'The ABC of channel regulation', *Cell*, vol. 82, no. 5, pp. 693-6.

Humphrey, SP & Williamson, RT (2001), 'A review of saliva: normal composition, flow, and function', *J Prosthet Dent*, vol. 85, no. 2, pp. 162-9.

Huq, M., Gonis, G & Istivan, T (2014), 'Development and Evaluation of a Multiplex PCR for the Detection of *Campylobacter concisus* and Other *Campylobacter* spp. from Gastroenteritis Cases', *OJMM*, vol. 4, pp. 29-37

Inglis, GD., Boras, VF & Houde, A (2011), 'Enteric campylobacteria and RNA viruses associated with healthy and diarrheic humans in the Chinook health region of southwestern Alberta, Canada', *J Clin Microbiol*, vol. 49, no. 1, pp. 209-19.

Inglis, GD., Morck, DW., Mcallister, TA., Entz, T., Olson, ME., Yanke, LJ & Read, RR (2006), 'Temporal prevalence of antimicrobial resistance in *Campylobacter* spp. from beef cattle in Alberta feedlots', *Appl Environ Microbiol*, vol. 72, no. 6, pp. 4088-95.

Ismail, Y., Lee, H., Riordan, SM., Grimm, MC & Zhang, L (2013), 'The effects of oral and enteric *Campylobacter concisus* strains on expression of TLR4, MD-2, TLR2, TLR5 and COX-2 in HT-29 cells', *PLoS One*, vol. 8, no. 2, p. e56888.

Ismail, Y., Mahendran, V., Octavia, S., Day, AS., Riordan, SM., Grimm, MC., Lan, R., Lemberg, D., Tran, TA & Zhang, L (2012), 'Investigation of the enteric pathogenic potential of oral *Campylobacter concisus* strains isolated from patients with inflammatory bowel disease', *PLoS One*, vol. 7, no. 5, p. e38217.

Istivan, T (2005), 'Molecular Characterisation of *Campylobacter concisus*: a potential etiological agent of gastroenteritis in children', RMIT University, Australia.

Istivan, T., Ward, P & Coloe , P (2010), '*Campylobacter concisus*: an emerging pathogen of the gastrointestinal tract', *Applied Microbiology and Microbial Biotechnology*, pp. 626-34.

Istivan, TS., Coloe, PJ., Fry, BN., Ward, P & Smith, SC (2004), 'Characterization of a haemolytic phospholipase A(2) activity in clinical isolates of *Campylobacter concisus*', *J Med Microbiol*, vol. 53, no. Pt 6, pp. 483-93.

Istivan, TS., Smith, SC., Fry, BN & Coloe, PJ (2008), 'Characterization of *Campylobacter concisus* hemolysins', *FEMS Immunol Med Microbiol*, vol. 54, no. 2, pp. 224-35.

Istivan, TS., Ward, PW., Lee, A., Coloe, PJ & Smith, SC (1998), 'Hemolysins of *Campylobacter concisus*', paper presented to In *Campylobacter, Helicobacter* and Related Organisms, Cape Town: University of Cape Town Press.

Javed, MA., Grant, AJ., Bagnall, MC., Maskell, DJ., Newell, DG & Manning, G (2010), 'Transposon mutagenesis in a hyper-invasive clinical isolate of *Campylobacter jejuni* reveals a number of genes with potential roles in invasion', *Microbiology*, vol. 156, no. 4, pp. 1134-43.

Johnson, CC & Finegold, SM (1987), 'Uncommonly encountered, motile, anaerobic gram-negative bacilli associated with infection', *Rev Infect Dis*, vol. 9, no. 6, pp. 1150-62.

Kaakoush, N., Deshpande, N., Wilkins, M., Raftery, M., Janitz, K & Mitchell, H (2011a), 'Comparative analyses of *Campylobacter concisus* strains reveal the genome of the reference strain BAA-1457 is not representative of the species', *Gut Pathog*, vol. 3, p. 15.

Kaakoush, N., Deshpande, N., Wilkins, M., Tan, C., Burgos-Portugal, J., Raftery, M., Day, A., Lemberg, D & Mitchell, H (2011b), 'The pathogenic potential of *Campylobacter concisus* strains associated with chronic intestinal diseases', *PLoS One*, vol. 6, no. 12, p. e29045.

Kaakoush, N., Man, SM., Lamb, S., Raftery, MJ., Wilkins, MR., Kovach, Z & Mitchell, H (2010), 'The secretome of *Campylobacter concisus*', *FEBS J*, vol. 277, no. 7, pp. 1606-17.

Kaakoush, NO & Mitchell, HM (2012), '*Campylobacter concisus* - A new player in intestinal disease', *Front Cell Infect Microbiol*, vol. 2, p. 4.

Kaakoush, NO., Sodhi, N., Chenu, JW., Cox, JM., Riordan, SM & Mitchell, HM (2014), 'The interplay between *Campylobacter* and *Helicobacter* species and other gastrointestinal microbiota of commercial broiler chickens', *Gut Pathog*, vol. 6, p. 18.

Kalischuk, LD & Inglis, GD (2011), 'Comparative genotypic and pathogenic examination of *Campylobacter concisus* isolates from diarrheic and non-diarrheic humans', *BMC Microbiol*, vol. 11, p. 53.

Kamma, JJ., Diamanti-Kipioti, A., Nakou, M & Mitsis, FJ (2000a), 'Profile of subgingival microbiota in children with mixed dentition', *Oral Microbiol Immunol*, vol. 15, no. 2, pp. 103-11.

Kamma, JJ., Diamanti-Kipioti, A., Nakou, M & Mitsis, FJ (2000b), 'Profile of subgingival microbiota in children with primary dentition', *J Periodontal Res*, vol. 35, no. 1, pp. 33-41.

Kamma, JJ & Nakou, M (1997), 'Subgingival microflora in smokers with early onset periodontitis', *Anaerobe*, vol. 3, no. 2-3, pp. 153-7.

Kamma, JJ., Nakou, M & Baehni, PC (1999), 'Clinical and microbiological characteristics of smokers with early onset periodontitis', *J Periodontal Res*, vol. 34, no. 1, pp. 25-33.

Kamma, JJ., Nakou, M & Manti, FA (1994), 'Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters', *J Periodontol*, vol. 65, no. 11, pp. 1073-8.

Kamma, JJ., Nakou, M & Persson, RG (2001), 'Association of early onset periodontitis microbiota with aspartate aminotransferase activity in gingival crevicular fluid', *J Clin Periodontol*, vol. 28, no. 12, pp. 1096-105.

Ketley, JM (1997), 'Pathogenesis of enteric infection by *Campylobacter*', *Microbiology*, vol. 143 (Pt 1), pp. 5-21.

Kirk, KF., Nielsen, HL & Nielsen, H (2015), 'The susceptibility of *Campylobacter concisus* to the bactericidal effects of normal human serum', *APMIS*, vol. 123, no. 3, pp. 269-74.

Konkel, ME., Kim, BJ., Klena, JD., Young, CR & Ziprin, R (1998), 'Characterization of the thermal stress response of *Campylobacter jejuni*', *Infect Immun*, vol. 66, no. 8, pp. 3666-72.

Kovach, Z., Kaakoush, NO., Lamb, S., Zhang, L., Raftery, MJ & Mitchell, H (2011), 'Immunoreactive proteins of *Campylobacter concisus*, an emergent intestinal pathogen', *FEMS Immunol Med Microbiol*, vol. 63, no. 3, pp. 387-96.

Lacroix, M (2008), 'Persistent use of “false” cell lines', *International journal of cancer*, vol. 122, no. 1, pp. 1-4.

Laemmli, UK (1970), 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', *Nature (London)*, vol. 227, pp. 680-5

Laniewski, P., Kuczkowski, M., Chrzastek, K., Wozniak, A., Wyszynska, A., Wieliczko, A & Jagusztyn-Krynicka, EK (2014), 'Evaluation of the immunogenicity of *Campylobacter jejuni* CjaA protein delivered by *Salmonella enterica* sv. Typhimurium strain with regulated delayed attenuation in chickens', *World J Microbiol Biotechnol*, vol. 30, no. 1, pp. 281-92.

Larson, CL., Christensen, JE., Pacheco, SA., Minnich, SA & Konkel, ME (2008), '*Campylobacter jejuni* secretes proteins via the flagellar Type III Secretion System that contribute to host cell invasion and gastroenteritis', *Campylobacter, 3rd Edition*, pp. 315-32.

Lastovica, A (2006), 'Emerging *Campylobacter* spp.: the tip of the iceberg', *Clin. Microbiol. Newsl.*, no. 28, pp. 49–56.

Lastovica, AJ (2009), 'Clinical relevance of *Campylobacter concisus* isolated from pediatric patients', *J Clin Microbiol*, vol. 47, no. 7, p. 2360.

Lastovica, AJ & Le Roux, E (2000), 'Efficient isolation of campylobacteria from stools', *J Clin Microbiol*, vol. 38, no. 7, pp. 2798-9.

Lauwers, SD, T. Van Etterijck, R *Et Al.* (1991), 'Isolation of *Campylobacter concisus* from human faeces.', *Microbial Ecology in Health and Diseases 4 (suppl).* p. 991.

Lavrencic, P., Kaakoush, NO., Huinao, KD., Kain, N & Mitchell, HM (2012), 'Investigation of motility and biofilm formation by intestinal *Campylobacter concisus* strains', *Gut Pathog*, vol. 4, no. 1, p. 22.

Lawson, AJ., Linton, D & Stanley, J (1998), '16S rRNA gene sequences of 'Candidatus *Campylobacter hominis*', a novel uncultivated species, are found in the gastrointestinal tract of healthy humans', *Microbiology*, vol. 144 (Pt 8), pp. 2063-71.

Leach, SA (1997), 'Growth, survival and pathogenicity of enteric campylobacters.', *Rev Med Microbiol*, no. 8, pp. 113-24.

Lee, H., Ma, R., Grimm, MC., Riordan, SM., Lan, R., Zhong, L., Raftery, M & Zhang, L (2014), 'Examination of the anaerobic growth of *Campylobacter concisus* strains', *Int J Microbiol*, vol. 2014, p. 476047.

Li, YP., Ingmer, H., Madsen, M & Bang, DD (2008), 'Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes', *BMC Microbiol*, vol. 8, p. 107.

Lindblom, GB., Sjogren, E., Hansson-Westerberg, J & Kaijser, B (1995), '*Campylobacter upsaliensis*, *C. sputorum sputorum* and *C. concisus* as common causes of diarrhoea in Swedish children', *Scand J Infect Dis*, vol. 27, no. 2, pp. 187-8.

Linton, D., Lawson, AJ., Owen, RJ & Stanley, J (1997), 'PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples', *J Clin Microbiol*, vol. 35, no. 10, pp. 2568-72.

Linton, D., Owen, RJ & Stanley, J (1996), 'Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals', *Res Microbiol*, vol. 147, no. 9, pp. 707-18.

Lynch, OA., Cagney, C., Mcdowell, DA & Duffy, G (2011), 'Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol', *Int J Food Microbiol*, vol. 150, no. 2-3, pp. 171-7.

Macfarlane, S., Furrie, E., Macfarlane, GT & Dillon, JF (2007), 'Microbial colonization of the upper gastrointestinal tract in patients with Barrett's esophagus', *Clin Infect Dis*, vol. 45, no. 1, pp. 29-38.

Macuch, PJ & Tanner, AC (2000), '*Campylobacter* species in health, gingivitis, and periodontitis', *J Dent Res*, vol. 79, no. 2, pp. 785-92.

Mahendran, V., Riordan, SM., Grimm, MC., Tran, TA., Major, J., Kaakoush, NO., Mitchell, H & Zhang, L (2011), 'Prevalence of *Campylobacter* species in adult Crohn's disease and the preferential colonization sites of *Campylobacter* species in the human intestine', *PLoS One*, vol. 6, no. 9, p. e25417.

Mahendran, V., Tan, YS., Riordan, SM., Grimm, MC., Day, AS., Lemberg, DA., Octavia, S., Lan, R & Zhang, L (2013), 'The prevalence and polymorphisms of zonula occluden toxin gene in multiple *Campylobacter concisus* strains isolated from saliva of patients with inflammatory bowel disease and controls', *PLoS One*, vol. 8, no. 9, p. e75525.

Maher, M., Finnegan, C., Collins, E., Ward, B., Carroll, C & Cormican, M (2003), 'Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of feces', *J Clin Microbiol*, vol. 41, no. 7, pp. 2980-6.

Malik-Kale, P., Parker, CT & Konkel, ME (2008), 'Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression', *J Bacteriol*, vol. 190, no. 7, pp. 2286-97.

Man, S., Kaakoush, N., Leach, S., Nahidi, L., Lu, HK., Norman, J., Day, AS., Zhang, L & Mitchell, H (2010a), 'Host attachment, invasion, and stimulation of proinflammatory cytokines by *Campylobacter concisus* and other non-*Campylobacter jejuni Campylobacter* species', *J Infect Dis*, vol. 202, no. 12, pp. 1855-65.

Man, S., Zhang, L., Day, A., Leach, S., Lemberg, D & Mitchell, H (2010b), '*Campylobacter concisus* and other *Campylobacter* species in children with newly diagnosed Crohn's disease', *Inflamm Bowel Dis*, vol. 16, no. 6, pp. 1008-16.

Man, SM (2011), 'The clinical importance of emerging *Campylobacter* species', *Nat Rev Gastroenterol Hepatol*, vol. 8, no. 12, pp. 669-85.

Man, SM., Zhang, L., Day, AS., Leach, S & Mitchell, H (2008), 'Detection of enterohepatic and gastric *Helicobacter* species in fecal specimens of children with Crohn's disease', *Helicobacter*, vol. 13, no. 4, pp. 234-8.

Mansell, TJ., Guarino, C & Delisa, MP (2013), 'Engineered genetic selection links in vivo protein folding and stability with asparagine-linked glycosylation', *Biotechnol J*, vol. 8, no. 12, pp. 1445-51.

Markwell, MA., Haas, SM., Bieber, LL & Tolbert, NE (1978), 'A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples', *Anal Biochem*, vol. 87, no. 1, pp. 206-10.

Marshall, DG., Dundon, WG., Beesley, SM & Smyth, CJ (1998), '*Helicobacter pylori*--a conundrum of genetic diversity', *Microbiology*, vol. 144 (Pt 11), pp. 2925-39.

Matsheka, MI., Elisha, BG., Lastovica, AL & On, SL (2002), 'Genetic heterogeneity of *Campylobacter concisus* determined by pulsed field gel electrophoresis-based macrorestriction profiling', *FEMS Microbiol Lett*, vol. 211, no. 1, pp. 17-22.

Matsheka, MI., Lastovica, AJ & Elisha, BG (2001), 'Molecular identification of *Campylobacter concisus*', *J Clin Microbiol*, vol. 39, no. 10, pp. 3684-9.

Matsheka, MI., Lastovica, AJ., Zappe, H & Elisha, BG (2006), 'The use of (GTG)5 oligonucleotide as an RAPD primer to type *Campylobacter concisus*', *Lett Appl Microbiol*, vol. 42, no. 6, pp. 600-5.

Miller, WG., Chapman, MH., Yee, E., On, SL., Mcnulty, DK., Lastovica, AJ., Carroll, AM., Mcnamara, EB., Duffy, G & Mandrell, RE (2012), 'Multilocus sequence typing methods for the emerging *Campylobacter* Species *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus*, and *C. curvus*', *Front Cell Infect Microbiol*, vol. 2, p. 45.

Moore, JE., Barton, MD., Blair, IS., Corcoran, D., Dooley, JS., Fanning, S., Kempf, I., Lastovica, AJ., Lowery, CJ., Matsuda, M., Mcdowell, DA., Mcmahon, A., Millar, BC., Rao, JR., Rooney, PJ., Seal, BS., Snelling, WJ & Tolba, O (2006), 'The epidemiology of antibiotic resistance in *Campylobacter*', *Microbes Infect*, vol. 8, no. 7, pp. 1955-66.

Moore, LV., Moore, WE., Cato, EP., Smibert, RM., Burmeister, JA., Best, AM & Ranney, RR (1987), 'Bacteriology of human gingivitis', *J Dent Res*, vol. 66, no. 5, pp. 989-95.

Mukhopadhya, I., Thomson, JM., Hansen, R., Berry, SH., El-Omar, EM & Hold, GL (2011), 'Detection of *Campylobacter concisus* and other *Campylobacter* species in colonic biopsies from adults with ulcerative colitis', *PLoS One*, vol. 6, no. 6, p. e21490.

Muller, A., Thomas, GH., Horler, R., Brannigan, JA., Blagova, E., Levdikov, VM., Fogg, MJ., Wilson, KS & Wilkinson, AJ (2005), 'An ATP-binding cassette-type cysteine transporter in *Campylobacter jejuni* inferred from the structure of an extracytoplasmic solute receptor protein', *Mol Microbiol*, vol. 57, no. 1, pp. 143-55.

Murch, SH., Braegger, CP., Walker-Smith, JA & Macdonald, TT (1993), 'Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease', *Gut*, vol. 34, no. 12, pp. 1705-9.

Murray, P., Rosenthal, K & Pfaller, M (2009), *Medical Microbiology*, Mosby Elsvier, Philadelphia.

Musmanno, RA., Russi, M., Figura, N., Guglielmetti, P., Zanchi, A., Signori, R & Rossolini, A (1998), 'Unusual species of campylobacters isolated in the Siena Tuscany area, Italy', *New Microbiol*, vol. 21, no. 1, pp. 15-22.

Muyzer, G., De Waal, EC & Uitterlinden, AG (1993), 'Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA', *Appl Environ Microbiol*, vol. 59, no. 3, pp. 695-700.

Muyzer, G & Smalla, K (1998), 'Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology', *Antonie Van Leeuwenhoek*, vol. 73, no. 1, pp. 127-41.

Nachamkin, I., Szymanski, CM & Blaser, MJ (2008), *Campylobacter*, No. Ed. 3, ASM Press.

Najdenski, H., Heyndrickx, M., Herman, L & Messens, W (2008), 'Fla-DGGE analysis of *Campylobacter jejuni* and *Campylobacter coli* in cecal samples of broilers without cultivation', *Vet Microbiol*, vol. 127, no. 1-2, pp. 196-202.

Nakou, M., Kamma, JJ., Andronikaki, A & Mitsis, F (1998), 'Subgingival microflora associated with nifedipine-induced gingival overgrowth', *Journal of periodontology*, vol. 69, no. 6, pp. 664-9.

Newell, DG (2005), '*Campylobacter concisus*: an emerging pathogen?', *Eur J Gastroenterol Hepatol*, vol. 17, no. 10, pp. 1013-4.

Nickerson, CA., Goodwin, TJ., Terlonge, J., Ott, CM., Buchanan, KL., Uicker, WC., Emami, K., Leblanc, CL., Ramamurthy, R & Clarke, MS (2001), 'Three-dimensional tissue assemblies: novel models for the study of *Salmonella enterica* serovar Typhimurium pathogenesis', *Infect Immun*, vol. 69, no. 11, pp. 7106-20.

Nielsen, H., Ejlertsen, T., Engberg, J & Nielsen, H (2011a), 'Clinical epidemiology and manifestations of *Campylobacter concisus*', *in European Societyof Clinical Microbiology and Infectious Diseases, Milan*, p. P990.

Nielsen, H., Ejlertsen, T., Engberg, J & Nielsen, H (2013a), 'High incidence of *Campylobacter concisus* in gastroenteritis in North Jutland, Denmark: a population-based study', *Clin Microbiol Infect*, vol. 19, no. 5, pp. 445-50.

Nielsen, H., Nielsen, H., Ejlertsen, T., Engberg, J., Gunzel, D., Zeitz, M., Hering, N., Fromm, M., Schulzke, J & Bucker, R (2011b), 'Oral and fecal *Campylobacter concisus* strains perturb barrier function by apoptosis induction in HT-29/B6 intestinal epithelial cells', *PLoS One*, vol. 6, no. 8, p. e23858.

Nielsen, HL., Engberg, J., Ejlertsen, T & Nielsen, H (2013b), 'Comparison of polycarbonate and cellulose acetate membrane filters for isolation of *Campylobacter concisus* from stool samples', *Diagn Microbiol Infect Dis*, vol. 76, no. 4, pp. 549-50.

O'rourke, JL., Grehan, M & Lee, A (2001), 'Non-*pylori Helicobacter* species in humans', *Gut*, vol. 49, no. 5, pp. 601-6.

On, S (2001), 'Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future propects and immediate concerns.', *Symp Ser Soc Appl Micrpbiolol.*, no. 30, p. Suppl 1:15.

Park, SF (2002), 'The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens', *Int J Food Microbiol*, vol. 74, no. 3, pp. 177-88.

Paster, BJ & Gibbons, RJ (1986), 'Chemotactic response to formate by *Campylobacter concisus* and its potential role in gingival colonization', *Infect Immun*, vol. 52, no. 2, pp. 378-83.

Pawelec, D., Jakubowska-Mroz, J & Jagusztyn-Krynicka, EK (1998), '*Campylobacter jejuni* 72Dz/92 *cjaC* gene coding 28 kDa immunopositive protein, a homologue of the solute-binding components of the ABC transport system', *Lett Appl Microbiol*, vol. 26, no. 1, pp. 69-76.

Pawelec, D., Korsak, D., Wyszynska, A., Rozynek, E., Popowski, J & Jagusztyn-Krynicka, E (2000), 'Genetic diversity of the *Campylobacter* genes coding immunodominant proteins', *FEMS Microbiol Lett*, vol. 185, no. 1, pp. 43-9.

Penner, JL (1988), 'The genus *Campylobacter*: a decade of progress', *Clin Microbiol Rev*, vol. 1, no. 2, pp. 157-72.

Pereira, VT., Pavan, P., Souza, RC., Souto, R., Vettore, MV., Torres, SR., Colombo, A., De Uzeda, M., Sansone, C & Goncalves, LS (2014), 'The association between detectable plasmatic HIV viral load and different subgingival microorganisms in HIV-infected Brazilian adults: a multilevel analysis', *J Periodontol*, vol. 85, no. 5, pp. 697-705.

Persson, S & Olsen, KE (2005), 'Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples', *J Med Microbiol*, vol. 54, no. Pt 11, pp. 1043-7.

Petersen, RF., Harrington, CS., Kortegaard, HE & On, SL (2007), 'A PCR-DGGE method for detection and identification of *Campylobacter*, *Helicobacter*, *Arcobacter* and related *Epsilobacteria* and its application to saliva samples from humans and domestic pets', *J Appl Microbiol*, vol. 103, no. 6, pp. 2601-15.

Pfaffl, MW (2001), 'A new mathematical model for relative quantification in real-time RT–PCR', *Nucleic Acids Res*, vol. 29, no. 9, pp. e45-e.

Podolsky, DK (2002), 'Inflammatory bowel disease', *N Engl J Med*, vol. 347, no. 6, pp. 417-29.

Pruckler, JM., Fields, PI & Fitzgerald, C (2002), 'Composite analysis of the phenotypic and genotypic characteristics of *C. concisus* strains', *J. Clin. Microbiol*, vol. 2001, p. P230.

Rossi, M., Debruyne, L., Zanoni, RG., Manfreda, G., Revez, J & Vandamme, P (2009), '*Campylobacter avium* sp. nov., a hippurate-positive species isolated from poultry', *Int J Syst Evol Microbiol*, vol. 59, no. 9, pp. 2364-9.

Russell, J & Ward, P (1998), 'Adhesion and invasion of HEp2 cells by *Campylobacter concisus* from children with diarrhoea', paper presented to *Campylobacter, Helicobacter* and Related Organisms, CapeTown.

Russell, RG., O'donnoghue, M., Blake, DC, Jr., Zulty, J & Detolla, LJ (1993), 'Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant Macaca mulatta', *J Infect Dis*, vol. 168, no. 1, pp. 210-5.

Saier, MH, Jr. (1994), 'Computer-aided analyses of transport protein sequences: gleaning evidence concerning function, structure, biogenesis, and evolution', *Microbiol Rev*, vol. 58, no. 1, pp. 71-93.

Sartor, RB (2008), 'Microbial influences in inflammatory bowel diseases', *Gastroenterology*, vol. 134, no. 2, pp. 577-94.

Scanlon, KA., Cagney, C., Walsh, D., Mcnulty, D., Carroll, A., Mcnamara, EB., Mcdowell, DA & Duffy, G (2013), 'Occurrence and characteristics of fastidious *Campylobacteraceae* species in porcine samples', *Int J Food Microbiol*, vol. 163, no. 1, pp. 6-13.

Schmidt, F & Schmidt, W (1985), 'Aminotransferases in human pathology and clinical chemistry ', *In: Christen P, Metzler DE, eds. Transaminases. New York: John Wiley & Sons*.

Sellars, MJ., Hall, SJ & Kelly, DJ (2002), 'Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen', *J Bacteriol*, vol. 184, no. 15, pp. 4187-96.

Serraino, A., Florio, D., Giacometti, F., Piva, S., Mion, D & Zanoni, RG (2013), 'Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy', *J Dairy Sci*, vol. 96, no. 5, pp. 2801-7.

Skirrow, MB., Jones, DM., Sutcliffe, E & Benjamin, J (1993), '*Campylobacter* bacteraemia in England and Wales, 1981-91', *Epidemiol Infect*, vol. 110, no. 3, pp. 567-73.

Skirrow, M (1992), 'Clinical epidemiologic considerations. In *Camplylobacter jejuni*, current status and future trends ed', *Nachampkin, I., Blaser, M.J and Tompkins, L.S*, pp. 3-8.

Socransky, SS., Haffajee, AD., Cugini, MA., Smith, C & Kent, RL. (1998), 'Microbial complexes in subgingival plaque', *J Clin Periodontol*, vol. 25, no. 2, pp. 134-44.

Sonnenberg, A (2010), 'Temporal changes in the age distribution of inflammatory bowel disease hospitalization: data from England and Scotland', *Eur J Gastroenterol Hepatol*, vol. 22, no. 1, pp. 95-101.

Sorvillo, FJ., Lieb, LE & Waterman, SH (1991), 'Incidence of campylobacteriosis among patients with AIDS in Los Angeles County', *J Acquir Immune Defic Syndr*, vol. 4, no. 6, pp. 598-602.

Stintzi, A (2003), 'Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation', *J Bacteriol*, vol. 185, no. 6, pp. 2009-16.

Szymanski, CM., Burr, DH & Guerry, P (2002), '*Campylobacter* protein glycosylation affects host cell interactions', *Infect Immun*, vol. 70, no. 4, pp. 2242-4.

Tam, R & Saier, M (1993), 'Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria', *Microbiol Rev*, vol. 57, no. 2, pp. 320-46.

Tankovic, J., Burghoffer, B & Petit, J (2009), 'Frequent detection by real-time PCR of bacteria from the *Helicobacter* and *Campylobacter* genera in stool samples from inflammatory bowel disease patients', paper presented to 19th European Congress of Clinical Microbiology and Infectious Diseases.

Tanner, A., Badger, S., Lai, C., Listgarten, M., Visconti, R & Socransky, S (1981), '*Wolinella* gen. nov., *Wolinella succinogenes* (Vibrio succinogenes Wolin *et al*.) comb. nov., and description of *Bateroides gracilis* sp.nov., *Wolinella recta* sp. nov., *Camylobacter concisus* sp. nov., and *Eikenella corrodens* from humans with periodontal disease', *Int J Syst Bacteriol*, no. 31, pp. 432-45.

Tanner, AC (1986), 'Characterization of *Wolinella* spp., *Campylobacter concisus*, *Bacteroides gracilis*, and *Eikenella corrodens* by polyacrylamide gel electrophoresis', *J Clin Microbiol*, vol. 24, no. 4, pp. 562-5.

Tanner, AC., Dzink, JL., Ebersole, JL & Socransky, SS (1987), '*Wolinella recta*, *Campylobacter concisus*, *Bacteroides gracilis*, and *Eikenella corrodens* from periodontal lesions', *J Periodontal Res*, vol. 22, no. 4, pp. 327-30.

Taubman, MA., Haffajee, AD., Socransky, SS., Smith, DJ & Ebersole, JL (1992), 'Longitudinal monitoring of humoral antibody in subjects with destructive periodontal diseases', *J Periodontal Res*, vol. 27, no. 5, pp. 511-21.

Tauxe, R (2001), 'Incidence, trends and source of Campylobacteriosis in developed countries: an overview. In: Proceedings of the inceasing incidence of Campylobacteriosis in Humans. Report and Proceedings of a WHO Consultaion of Experts,' *WHO, Geneva, Switzerland.*, pp. PP:42-3.

Tauxe, RV., Patton, CM., Edmonds, P., Barrett, TJ., Brenner, D & Blake, P (1985), 'Illness associated with *Campylobacter laridis*, a newly recognized *Campylobacter* species', *J Clin Microbiol*, vol. 21, no. 2, pp. 222-5.

Tauxe, RV., Pegues, DA & Hargrett-Bean, N (1987), '*Campylobacter* infections: the emerging national pattern', *Am J Public Health*, vol. 77, no. 9, pp. 1219-21.

Tu, ZC., Eisner, W., Kreiswirth, BN & Blaser, MJ (2005), 'Genetic divergence of *Campylobacter fetus* strains of mammal and reptile origins', *J Clin Microbiol*, vol. 43, no. 7, pp. 3334-40.

Uzzau, S & Fasano, A (2000), 'Cross-talk between enteric pathogens and the intestine', *Cell Microbiol*, vol. 2, no. 2, pp. 83-9.

Valenza, G., Frosch, M & Abele-Horn, M (2010), 'Antimicrobial susceptibility of clinical *Campylobacter* isolates collected at a German university hospital during the period 2006-2008', *Scand J Infect Dis*, vol. 42, no. 1, pp. 57-60.

Van Etterijck, R., Breynaert, J., Revets, H., Devreker, T., Vandenplas, Y., Vandamme, P & Lauwers, S (1996), 'Isolation of *Campylobacter concisus* from feces of children with and without diarrhea', *J Clin Microbiol*, vol. 34, no. 9, pp. 2304-6.

Van Vliet, AH & Ketley, JM (2001), 'Pathogenesis of enteric *Campylobacter* infection', *Symp Ser Soc Appl Microbiol*, no. 30, pp. 45S-56S.

Vanamburg (2014), *Colonoscopy/Endoscopy*, http://www.vanamburgsurgery.com/colonoscopy/, (2015).

Vandamme, P., Dewhirst, FE., Paster, BJ & On, S (2005), '*Campyobacter*, Bergey's Manual of Systematic Bacteriology eds D.J.Brenner, N.R.Krieg, and J.T.Staley', *New York:Springe*, vol. , no. , pp. 1147–60.

Vandamme, P., Falsen, E., Pot, B., Hoste, B., Kersters, K & De Ley, J (1989), 'Identification of EF group 22 campylobacters from gastroenteritis cases as *Campylobacter concisus*', *J Clin Microbiol*, vol. 27, no. 8, pp. 1775-81.

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, JP & Dediste, A (2006), 'Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium', *J Antimicrob Chemother*, vol. 57, no. 5, pp. 908-13.

Von Rosenvinge, EC., Song, Y., White, JR., Maddox, C., Blanchard, T & Fricke, WF (2013), 'Immune status, antibiotic medication and pH are associated with changes in the stomach fluid microbiota', *ISME J*, vol. 7, no. 7, pp. 1354-66.

Wilson, J., Hair, C., Knight, R., Catto-Smith, A., Bell, S., Kamm, M., Desmond, P., Mcneil, J & Connell, W (2010), 'High incidence of inflammatory bowel disease in Australia: a prospective population-based Australian incidence study', *Inflamm Bowel Dis*, vol. 16, no. 9, pp. 1550-6.

Wogahn, B (2015), *Colon cancer more common than you think*, http://www.oakleafmedical.com/hv/2012_wi/wi2012_coloncancer.php, (2015).

Wright, JA., Grant, AJ., Hurd, D., Harrison, M., Guccione, EJ., Kelly, DJ & Maskell, DJ (2009), 'Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch', *Microbiology*, vol. 155, no. Pt 1, pp. 80-94.

Wyszynska, A., Raczko, A., Lis, M & Jagusztyn-Krynicka, EK (2004), 'Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cjaA* gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*', *Vaccine*, vol. 22, no. 11-12, pp. 1379-89.

Wyszynska, A., Tomczyk, K & Jagusztyn-Krynicka, EK (2007), 'Comparison of the localization and post-translational modification of *Campylobacter coli* CjaC and its homolog from *Campylobacter jejuni*, Cj0734c/HisJ', *Acta Biochim Pol*, vol. 54, no. 1, pp. 143-50.

Wyszynska, A., Zycka, J., Godlewska, R & Jagusztyn-Krynicka, EK (2008), 'The *Campylobacter jejuni*/*coli cjaA* (*cj0982c*) gene encodes an N-glycosylated lipoprotein localized in the inner membrane', *Curr Microbiol*, vol. 57, no. 3, pp. 181-8.

Zhang, L., Budiman, V., Day, AS., Mitchell, H., Lemberg, DA., Riordan, SM., Grimm, M., Leach, ST & Ismail, Y (2010), 'Isolation and detection of *Campylobacter concisus* from saliva of healthy individuals and patients with inflammatory bowel disease', *J Clin Microbiol*, vol. 48, no. 8, pp. 2965-7.

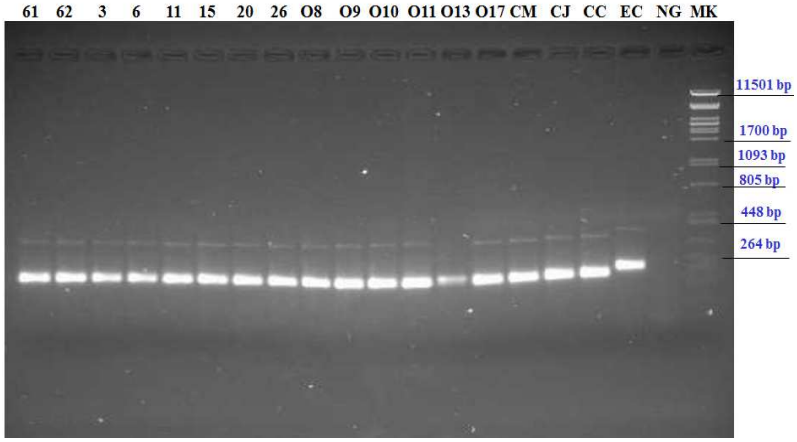
Zhang, L., Day, A., Mckenzie, G & Mitchell, H (2006), 'Nongastric *Helicobacter* species detected in the intestinal tract of children', *J Clin Microbiol*, vol. 44, no. 6, pp. 2276-9.

Zhang, L., Lee, H., Grimm, MC., Riordan, SM., Day, AS & Lemberg, DA (2014), '*Campylobacter concisus* and inflammatory bowel disease', *World J Gastroenterol*, vol. 20, no. 5, pp. 1259-67.

Zhang, L., Man, SM., Day, AS., Leach, ST., Lemberg, DA., Dutt, S., Stormon, M., Otley, A., O'loughlin, EV., Magoffin, A., Ng, PH & Mitchell, H (2009), 'Detection and isolation of *Campylobacter* species other than *C. jejuni* from children with Crohn's disease', *J Clin Microbiol*, vol. 47, no. 2, pp. 453-5.

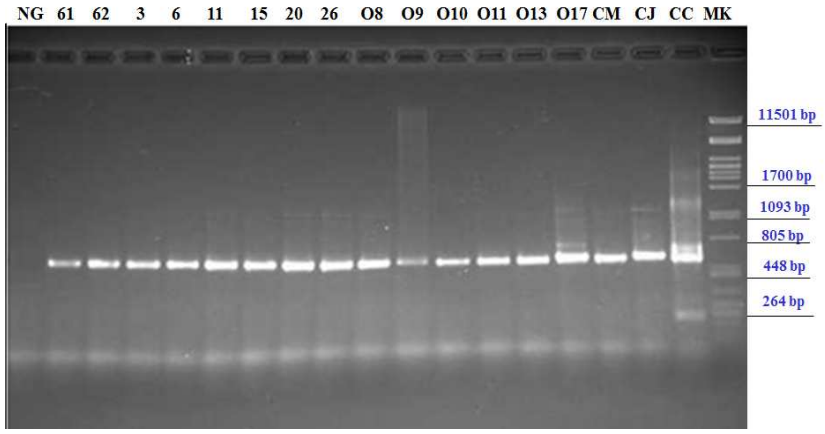
Appendices

Appendix I: PCR products obtained from amplifying the 16S rDNA gene of clinical and oral *C. concisus* isolates using Muyzer primer set 1.



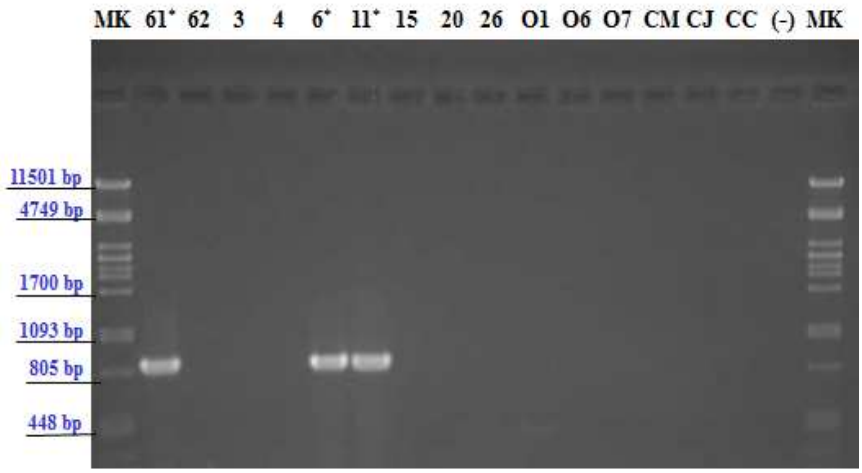
PCR products (170 bp) were loaded on a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. MK: lambda DNA marker. 61 & 62: *C. concisus* (ATCC 51561 & 51562). 3, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264). CJ: *C. jejuni* (81116). CC: *C. coli* (NCTC 11366). O: oral isolates.

Appendix II: PCR products obtained from amplifying the 16S rDNA gene of clinical and oral *C. concisus* isolates using Muyzer primer set 2.

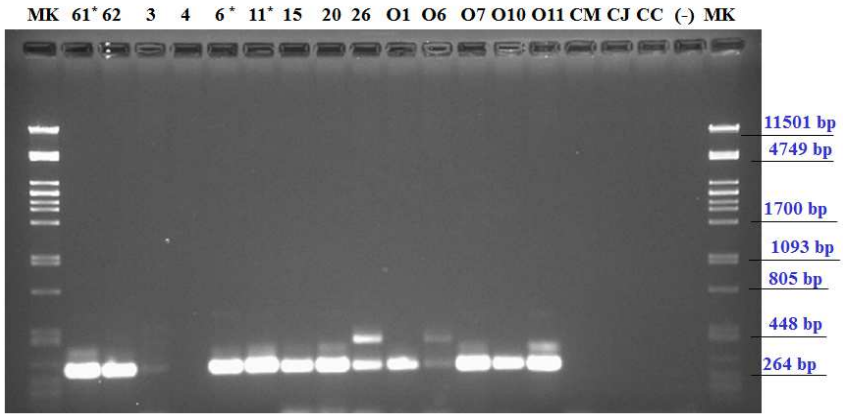


PCR products (562 bp) were loaded on a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. MK: lambda DNA marker. NG: negative control. 61 & 62: *C. concisus* (ATCC 51561 &51562). 3, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264), CJ: *C. jejuni* (81116), CC: *C. coli* (NCTC 11366). O: oral isolates.

Appendix III: PCR products obtained from amplifying *cjaC* of clinical and oral *C. concisus* isolates using primer set I.



Appendix IV: PCR products obtained from amplifying *cjaC* of clinical and oral *C. concisus* isolates using primer set II.



Appendices III and IV, the size of PCR products in Appendix III is 800 bp. The size of PCR products in Appendix IV is 300 bp MK: Lambda *Pst*I DNA ladder. 61& 62: *C. concisus*; (ATCC 51561 & 51562). 3, 4, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264), CJ: *C. jejuni* (81116), CC: *C. coli* (NCTC 11366). O: oral isolates. A & B: genomospecies. (-): no template control. *: genomospecies B. bp: base pair.

Appendix V: Nucleotide sequence of a PCR product obtained from amplifying *cjaC* of *C. concisus* ATCC 51561 using primer set I (746 bp).

CCTTAGTATTCACATCTCTTGCCACTTCAGCAGCAGCCGCTTCAAAGATAGCTTTTAGATCATCTGG
AAGTTTTTCATATGCATTTTTGTTGAAGAAAAACTGAGTTTCGCCATCTGGCTCTTGCCAGCCTGTG
TAGTAGTATTTTGCCACTTTGTGAAAGCCAAGTGCCATGTCATAAGCTGGGCTAACCCACTCGACT
GCATCGATAGTGCCCATCTCAAGAGCCATGTAAAGCTCGCCAGTTGGGATAGTGTTGATGTTTGCG
CCAAGTTTAGCGTAAATTTCACCGCCAAAGCCCGGAATTCTTATCTTTAAGCCTTTGATATCATCAA
CTGACTTGATCTCTTTTTTAAACCAGCCGCCCATTTGCATGCCAGTGTTACCAGCTCTAAAAATTTT
GATGTTATATGGGTCAAAAACTTTCGCCTCTAGCTCTTTGCCGCCGCCAAATTCATACCAAGCTGTT
TGCTCGTCGGTATTCATCATAAATGGTGTCGCTGTAAAAAACATAGTCTTAGGGTCTTTGCCCTTGT
AGTAGTAGCTAGCTGTGTAGCCGATGTCGTACTGACCGCTCTTTGCAAAGTCAAGCATCGCAAAAG
GTGACTTGTGCTTTGACGGGTAGTCGATCCTGATCTCGATGCGGCCGTTACTCATCTTTTCGACCTT
CTCTTTTAGCTCTTTTGGCACGTCACCAAGCACTGGCATAGTGCTCTCCCATGAGGTTGCAAGCTTG
AGCTTATAGACT

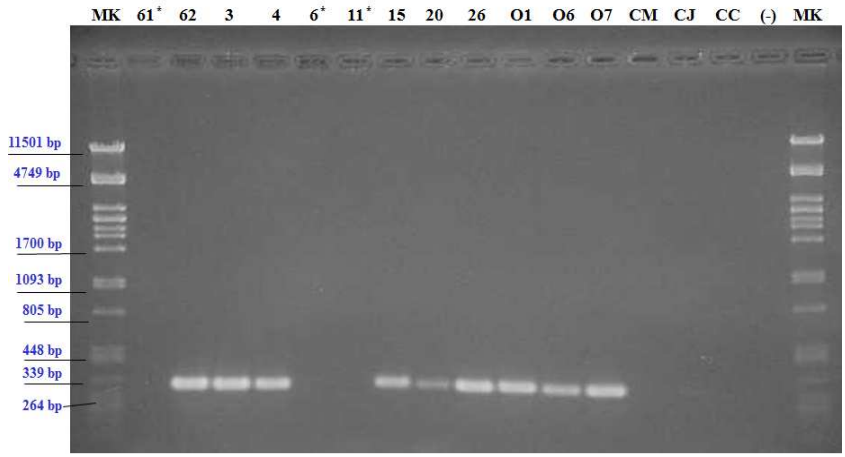
Appendix VI: Nucleotide sequence of a PCR product obtained from amplifying *cjaC* of *C. concisus* RCH6 using primer set I (746 bp).

CCTTAGTATTTACATCTCTTGCCACTTCTGCAGCAGCCGCTTCAAAGATAGCTTTTAGATCATCTGG
AAGTTTTTCATACGCGTTCTTATTGAAGAAAAACTGAGTTTCGCCATCTGGCTCTTGCCAGCCTGTG
TAGTAGTATTTTGCCACTTTGTGAAAGCCAAGTGCCATGTCATAAGCCGGGCTAACCCACTCGACC
GCGTCGATCGTACCCATCTCAAGTGCCATATAAAGCTCGCCAGTTGGGATAGTGTTGATATTTGCG
CCAAGTTTAGCGTAAATTTCACCGCCAAAGCCTGGAATTCTGATCTTTAAACCTTTGATATCATCA
ACTGACTTGATCTCTTTTTTAAACCAGCCGCCCATTTGCATGCCAGTGTTGCCAGCTCTAAAAATTT
TGATGTTATATGGATCAAAAACTTTGGCTTCTAGCTCTTTGCCACCACCAAATTCATACCAAGCTGT
TTGCTCGTCGGTATTCATCATAAATGGTGTTGCTGTAAAGAACATAGTCTTAGGATCTTTGCCTTTG
TAGTAGTAGCTAGCTGTGTAGCCGATATCGTACTGACCGCTCTTTGCAAAGTCAAGCATCGCAAAA
GGTGACTTGTGCTTTGACGGGTAGTCGATCCTGATCTCGATGCGGCCGTTACTCATCTTTTCGACCT
TCTCTTTTAGCTCTTTTGGCACGTCACCAAGCACTGGCATAGTGCTCTCCCATGAGCTTGCAAGCTT
GAGCTTATAGACT

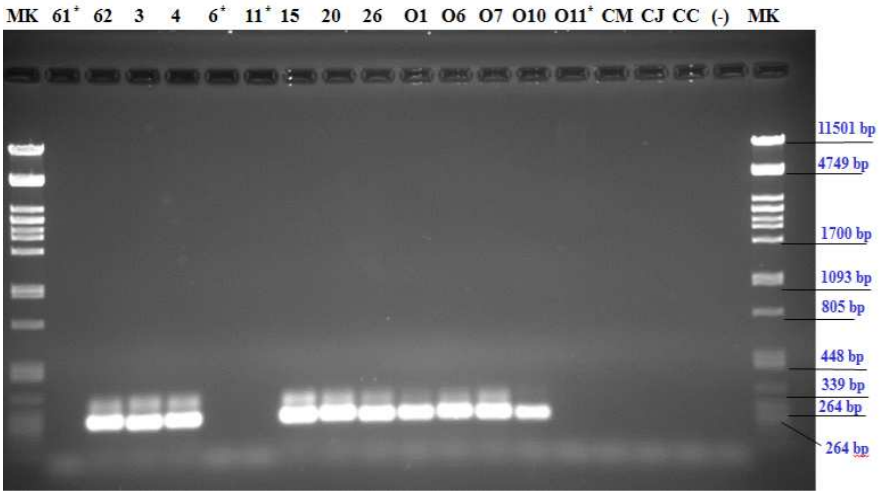
Appendix VII: Nucleotide sequence of a PCR product obtained from amplifying *cjaC* of *C. concisus* ATCC 51562 using primer set II (295 bp).

GCCAAGCGCCATGTCATAAGCTGGGCTAACCCATTCGACTGAGTCAATCGTTCCCATCTCAAGAGC
CATGTAAAGCTCACCAGTTGGGATAGTGTTAATGTTAGCGCCAAGTTTAGCGTAAATTTCACCACC
AAAGCCCGGAATTCTTATCTTTAAACCTTTGATATCATCTAGTGATTTTATCTCTTTTTTAAACCAG
CCACCCATTTGCATGCCGGTATTTCCAGCTCTAAAAATTTTGATATTGTATGGATCGTAAACTTTTG
CCTCAAGCTCTTTGCCGCCGCCAAATTCA

Appendix VIII: PCR products obtained from amplifying *cjaC* of *C. concisus* clinical and oral isolates using primer set III.

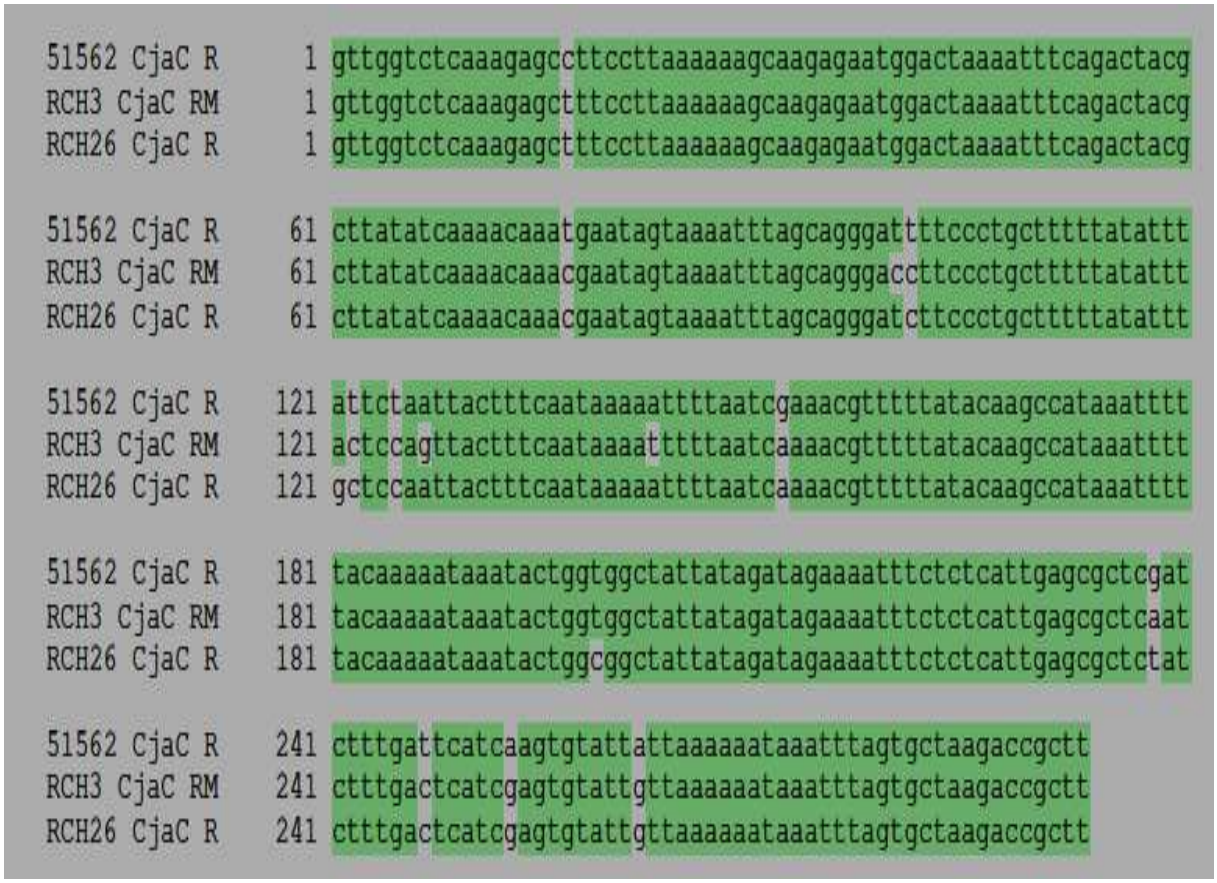


Appendix IX: PCR products obtained from amplifying *cjaC* of clinical and oral *C. concisus* isolates using primer set IV.

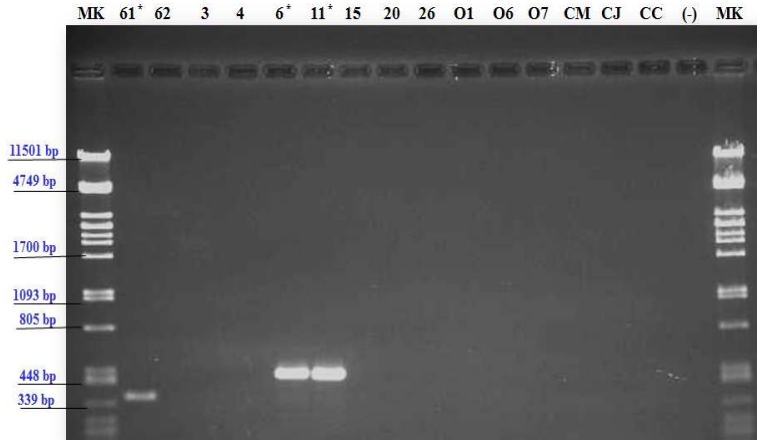


Appendices VIII and IX, the size of PCR product in Appendix VIII is 330 bp. The size of PCR products in Appendix IX is 270 bp. Lambda *Pst*I DNA ladder. 61& 62: *C. concisus*: (ATCC 51561 & 51562). 3, 4, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264), CJ; *C. jejuni* (81116), CC: *C. coli* (NCTC 11366). O; oral isolates. A & B: genomospecies. *: genomospecies B. (-): no template control bp: base pair.

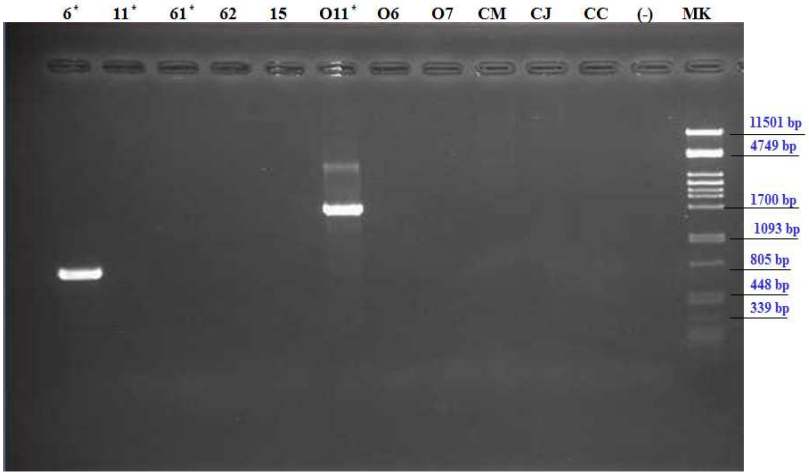
Appendix X: The alignment of *cjaC* nucleotide sequences obtained from PCR products obtained from *C. concisus* strains (51562, RCH3 and RCH26) using primer set III.



Appendix XI: PCR products obtained from amplifying *cjaC* and its adjacent gene (CCC13826_0962) of *C. concisus* clinical and oral isolates using primer set V.



Appendix XII: PCR products obtained from amplifying *cjaC* and its adjacent gene (CCC13826_0962) of *C. concisus* clinical and oral isolates using primer set VI.

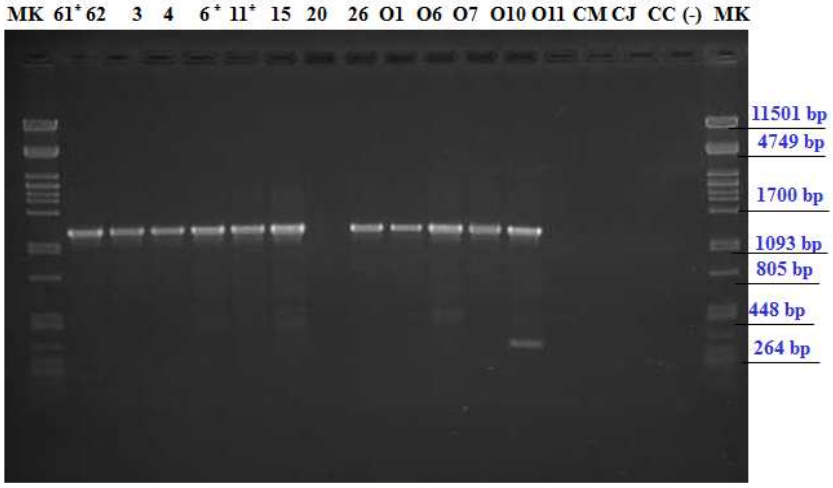


Appendices XI and XII, the size of PCR product in Appendix XI is 514 bp except strain 51561 was 330 bp. The size of PCR product in Appendix XII is 600 and 1700 bp. MK: Lambda *Pst*I DNA ladder. 61 & 62: *C. concisus*; (ATCC 51561 & 51562). 3, 4, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264), CJ: *C. jejuni* (81116), CC: *C. coli* (NCTC 11366). O: oral isolates. A & B: genomospecies. *: genomospecies B. (-): no template control. bp: base pair.

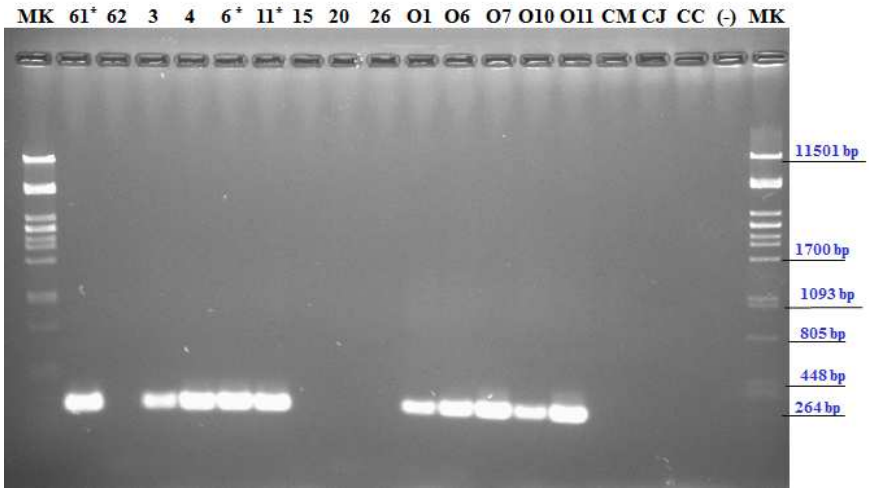
Appendix XIII: Nucleotide sequences of a PCR product obtain from amplifying *cjaC* and its adjacent gene (CCC13826_0962) of *C. concisus* O11 using primer set VI (1401 of 1700 bp).

ATCTCCTTCTCTAGCTTCTCATCAAATACATAAACACCACCATATTTTTTAGCAATGTTCTCTTGCTC
GCTAGTTAGATCAGAAATTCTTATAAAGCCATTAAAATAAGCAAGTGGTAGAAGTATAAATAAGC
AGATAAAAACTATAAATTTGACTTTTTTCATCATAAATCCTTTTAAATTTAAAACTATACAAGATA
GAAATTTCTCATAATCTTATTGTGAATATATTTATAATATTATTAATTTATTATGCTTAGCTAGGTTT
ATTAATTATAAAATTTATCCGAAAAACTCCAAATTTCATTTTCTTCTATATACTCAAAAAAAGCATA
AGCAACATCGGCTATATCAGCATAAACCCTTATTTTATCTACCATCTCTTTATTGTTAAATTTTTAA
GAGTGTTCATTTATTTATCCTTGTCTTTATTGCTGATTTTTTGAAATTTATTGTCTGCTAGATAAAAT
TTATTATCTCCAATTAGTCTTTGAGTGCTTTCTTTGGTAAATTTTATTCCACTTGCTTCATCGCCATA
TAGCCCAAATGTAACTTCTGTATAAAGCGTAGAAACTCCTATACTTATAGTATAATAGTGTCATTG
TCTATATAGAAATATCCTAAATTTATACTTAGATAGTTTTCATATTTATCATAATCTTTACCCATAA
ATTCTTTTAGTTTTTCTTCGTAGTTATTTAAAATTTTGTAGTTATCTATTTTTATATTGTCTTTTCTTG
AGTAGTATCTTTTTCCATTAGATAAAACTCTAGGTAATTTTTCATTAACTGGCGTCATATCAACTGC
ATAACGATTTTCGGCTAATTTTTTACCACTTAAGCCTTTTGTCATTATTCGTCTCTCTTCTTCTCTCTT
GTCTATCTCCTTCTCTAGCTTCTCATCAAATACATAAACACCACCATACTTTTTAGCAATGCTCTCT
TGTTCGCTAGTTAGATCAGAAATTCTTATGAAGCCATTAAAATAAGCAAGTGGTAGCAGTATAAAC
AAGCAGATAAAAACTATAAATTTGACTTTTTTCATCATAAATCCTTTTGAATTTTCACTATTGTCTT
ATCTTTTGCTTTAAAGTCTTATTGTGATTAGTTTTTGTGGTGGTTTTAAATTTTAAAAAGCAGGGAG
AGCTCCCTGCTGTGGTTATTTCTCTTCGTTTGTTTTGATGTAAGCGTAGTCAGAAATTTTAGTCCAC
TCTCTTGCTTTTTTGAGGAAAGCTCTTTGAGACTCGACGATCTCTTTAAATAGTGGATCTTTTGCGC
TCTCTTCATCAAGTAGCTCGTTTGTAGCCTTTTTAAGAGCTGCGATGACCTCTGGTGGGAAAGATTT
TACTTGGATGTCTGGATACTCAGCCTTCATCTTGTCCCAGTACTCTACGTTGCGTAAA

Appendix XIV: PCR products obtained from amplifying the DNA region between *cjaA* and the adjacent gene encodes glutamine transport ATP-binding protein GlnQ of clinical and oral *C. concisus* isolates using primer set CjaA.

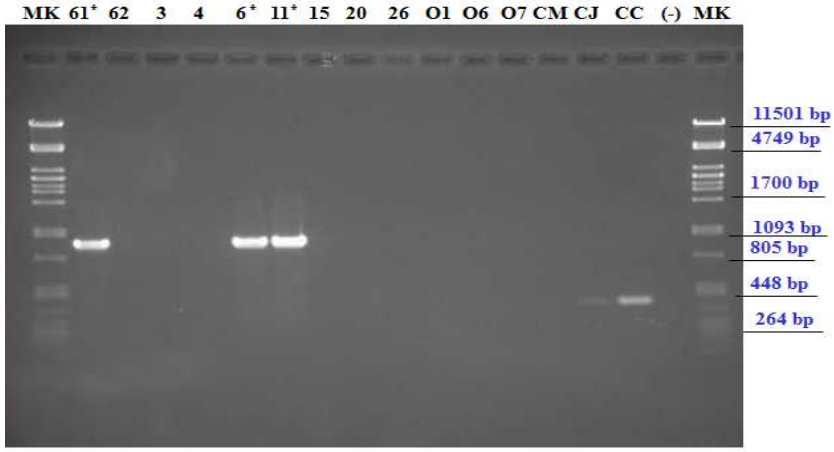


Appendix XV: PCR products obtained from amplifying *cjaA* of clinical and oral *C. concisus* isolates using CjaAT primer.

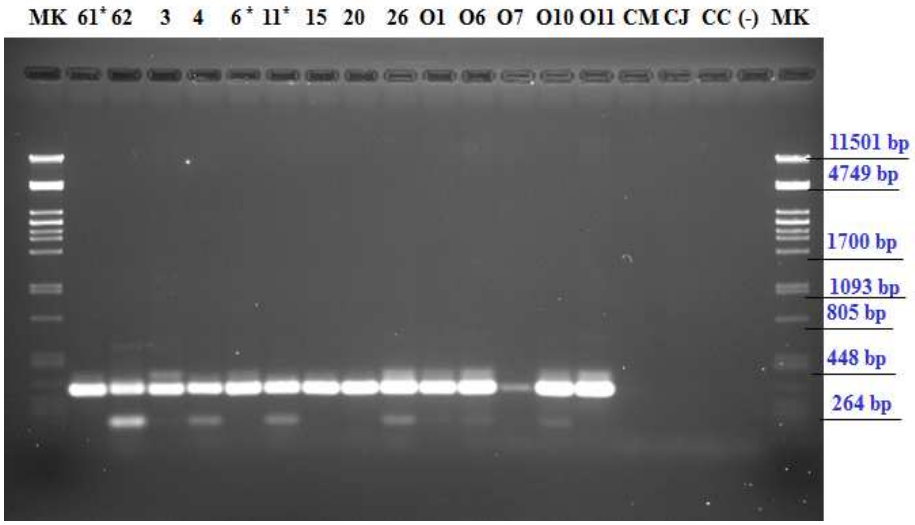


Appendices XIV and XV, the size of PCR product in Appendix XIV is 1200 bp. The size of PCR product in Appendix XV is 299 bp. PCR products were loaded on a 1.5% (w/v) agarose gel for electrophoresis. It was then stained in ethidium bromide and visualised by a UV trans-illuminator. MK: lambda DNA marker. 61 & 62: *C. concisus* (ATCC 51561 &51562). 3, 4, 6, 11, 15 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264), CJ: *C. jejuni* (81116), CC: *C. coli* (NCTC 11366). O: oral isolates.

Appendix XVI: PCR products obtained from amplifying *dnaJ* of *C. concisus* clinical and oral isolates using primer set DnaJ.

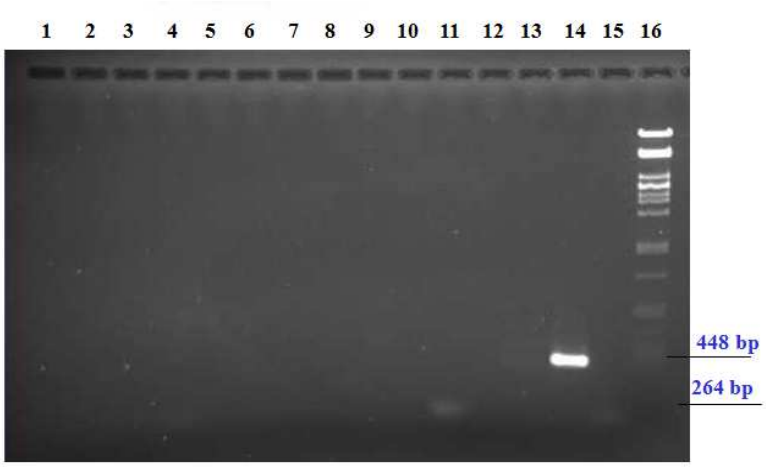


Appendix XVII: PCR products obtained from amplifying *dnaJ* of *C. concisus* clinical and oral isolates using primer set DnaJT.

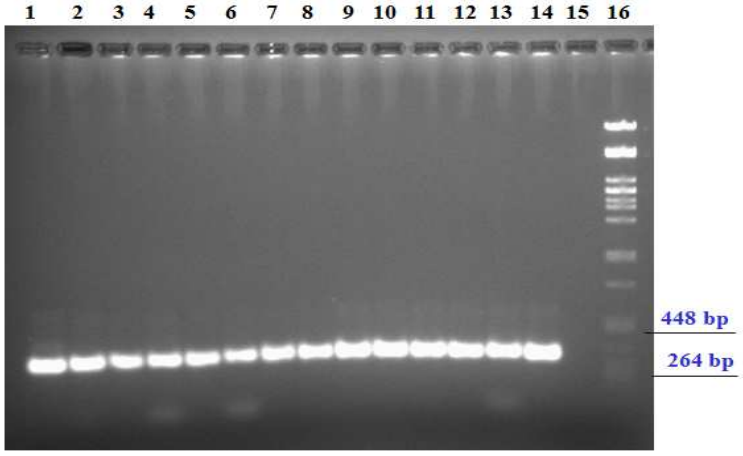


Appendices XVI and XVII, the size of PCR product in Appendix XVI is 900 bp. The size of PCR product in Appendix XVII is 304 bp. MK: Lambda *Pst*I DNA ladder. 61 & 62: *C. concisus* (ATCC 51561 & 51562). 3, 4, 6, 11, 15, 20 & 26: RCH isolates. CM: *C. mucosalis* (ATCC 43264). CJ: *C. jejuni* (81116). CC: *C. coli* (NCTC 11366). O: oral isolates. A & B: genomospecies. *: genomospecies B. (-): no template control. bp: base pair.

Appendix XVIII: The cDNA amplification of *atpA* (the housekeeping gene) using Semi-quantitative SYBR green assay.



A- RT-PCR products obtained from amplifying RNA of *atpA* (300 bp) in *C. concisus* ATCC 51561 using primer sets for AtpA. Lanes 1-3: triplicates of bacterial RNA from cells grown on HBA. Lanes 4-6: triplicates of bacterial RNA from cells grown on CA. Lanes 7-9: triplicates of bacterial RNA from cells maintained to INT407+DMEM. Lanes 10-12: triplicates of bacterial RNA from cells maintained DMEM. Lane 13: RNA extracted from INT407+DMEM without the bacteria. Lane 14: genomic DNA (control positive). Lane 15: non-template control. Lanes 16: lambda DNA marker.



B- RT-PCR amplification of cDNA obtained from *atpA* in *C. concisus* ATCC 51561 using primer set AtpA. The PCR products size is 300 bp. Lanes 1-3: triplicates of bacterial cDNA of cells grown on HBA. Lanes 4-6: triplicates of bacterial cDNA of cells grown on CA. Lanes 7-9: triplicates of bacterial cDNA from cells maintained in INT407+DMEM. Lanes 10-12: triplicates of bacterial cDNA of cells maintained DMEM. Lanes 13-14: genomic DNA (control positive). Lane 15: non-template control. Lanes 16: lambda DNA marker.

Appendix XIX: Coding and history of IBD patients.

RMIT code	Disease	Austin code	Age	Sex	IBD period	Gum	Smoking	Treatments
AUS1	UC	2JT	59	F	20	---	---	Crestor & salazopyrin
AUS2	CD	8TP	33	M	---	---	yes	Prednisolone
AUS3	CD	5SZ	30	F	7	---	yes	Pentasa: anti-inflammatory
AUS4	CD	7DW	49	M	17	---	yes	Prednisolone
AUS5	CD	12AH	63	F	---	gingivitis	---	Mesalazine
AUS6	CD	3DC	48	M	4	---	---	Methotrexate
AUS7	UC	10MS	27	M	11	---	---	prednisolone
AUS8	CD	13DV	34	M	21	---	---	Mesalazine , Imuran (immunosuppressive)
AUS9	CD	15KRA	33	M	31	---	yes	Somac, Imuran, VtiD
AUS10	CD	17ML	73	F	34	gingivitis	---	Azathioprine
AUS12	CD	20CR	49	F	5	---	---	Azathioprine, pentasa
AUS13	CD	21NB	48	F	11	---	---	Nexium, motilium
AUS14	CD	22RH	44	F	4	---	yes	Folic acid
AUS19	UC	30EC	27	F	6	---	---	pentasa
AUS20	UC	JM28	81	F	---	---	---	prednisolone
AUS21	UC	RW29	54	M	19	---	---	mesalazine
AUS22	UC	NV30	33	F	5	---	---	methotrexate
AUS23	CD	SDB31	22	F	---	---	---	---
AUS27	CD	MA36	42	M	10	bleeding	yes	Azathioprine
AUS28	UC	BC35	20	F	4	---	---	Mesalazine & imuran
AUS29	CD	RF39	47	M	---	---	yes	Symbicort, zoloft
AUS32	CD	FK34	70	F	6	---	---	---
AUS31	UC	HW42	22	M	20	---	---	Somac, Imuran & VtiD
AUS33	CD	CH44	40	M	28	---	yes	---
AUS34	CD	45LH	44	F	17	---	---	---
AUS36	CD	BS46	66	F	3	---	yes	Azathioprine, prednisolone
AUS37	UC	MA50	70	M	---	---	---	azathioprine
AUS39	CD	JD52	34	M	10	---	---	Imuran, salofalk, Diamox, Vytorin?
AUS41	CD	TM57	52	F	---	---	yes	Colazide, micardis
AUS42	CD	RD	61	F	11	---	yes	---
AUS45	UC	RI36	103	M	52	---	---	Azathioprine & pentasa
AUS46	CD	VL64	32	F	16	---	---	Azathioprine, pentasa
AUS48	UC	MS67	57	M	---	---	---	Nexium & motilium
AUS49	CD	AK68	27	F	4	---	---	---
AUS50	CD	DF69	43	M	---	---	yes	---
AUS51	CD	KH70	44	F	18	---	---	---
AUS52	CD	MC71	39	F	22	---	---	Azathioprine, colazal, valcyte

Appendix XX: Coding and history of control participants.

RMIT code	Austin code	Age	Sex	Gum	smoking	Treatments
AUS11	19JH	82	M	---	---	Nexium & motilium
AUS15	18SC	38	M	---	---	folic acid
AUS17	RT23	28	M	---	---	Azathioprine
AUS18	GL24	63	F	---	---	symbicort & zoloft
AUS24	NR32	77	M	---	---	---
AUS25	MA33	48	M	---	---	---
AUS26	BM34	26	F	---	---	---
AUS30	WS40	79	M	---	---	Azathioprine & prednisolone
AUS35	KT47	65	M	---	---	Imuran, salofalk, Diamox & vytorin
AUS38	PMC51	53	M	---	---	Colazide & micardis
AUS40	CL53	50	F	---	---	---
AUS43	FL61	67	M	---	---	Azathioprine & pentasa
AUS44	FN	78	F	---	---	---
AUS47	AB65	58	M	---	---	---

Appendix XXI: *C. concisus* isolates obtained from clinical samples (biopsies, faeces and gum) of IBD patients and control participants, and primer sets were used for confirming and typing these isolates.

Participant code	Isolate code	*Cam*. spp primers	Pcisus primers	CON1 primers	CON2 primers	VI primers	ZoT primers
AUS1	AUS1-Ga	1	1	0	1	1 L	0
AUS1	AUS1-Gb	1	1	0	1	1 L	0
AUS4	AUS4-Ga	1	1	0	1	0	0
AUS4	AUS4-Gb	1	1	0	1	0	0
AUS4	AUS4-Gc	1	1	0	1	0	0
AUS4	AUS4-Gg	1	1	0	1	0	0
AUS4	AUS4-Gl	1	1	0	1	0	0
AUS4	AUS4-Gk	1	1	0	1	0	0
AUS4	AUS4-Gm	1	1	0	1	0	0
AUS4	AUS4-Gn	1	1	0	1	0	0
AUS4	AUS4-Sf	1	1	0	1	0	0
AUS5	AUS5-Ga	1	1	1	0	NA	0
AUS5	AUS5-Gc	1	1	1	0	NA	0
AUS5	AUS5-Gd	1	1	0	1	0	0
AUS5	AUS5-Gf	1	1	0	1	0	0
AUS5	AUS5-Ge	1	1	0	1	0	0
AUS5	AUS5-Sa	1	1	1	0	NA	0
AUS5	AUS5-Sb	1	1	0	1	0	0
AUS5	AUS5-Sc	1	1	1	0	NA	0
AUS5	AUS5-Sm	1	1	1	0	NA	0
AUS5	AUS5-Sd	1	1	0	1	0	0
AUS5	AUS5-Sf	1	1	1	0	NA	0
AUS6	AUS6-Ga	1	1	0	1	1	0
AUS6	AUS6-Ga	1	1	0	1	1	0
AUS7	AUS7-Ga	1	1	1	0	NA	1
AUS7	AUS7-Gb	1	1	0	1	0	0
AUS7	AUS7-Gc	1	1	0	1	0	0
AUS7	AUS7-Gd	1	1	0	1	1 L	0
AUS9	AUS9-Ga	1	1	0	1	0	0
AUS9	AUS9-Gb	1	1	0	1	0	0
AUS9	AUS9-Gc	1	1	0	1	0	0
AUS9	AUS9-Gd	1	1	0	1	0	0
AUS9	AUS9-Ge	1	1	0	1	0	0
AUS9	AUS9-Gg	1	1	0	1	0	0
AUS9	AUS9-GL	1	1	0	1	0	0
AUS9	AUS9-Gk	1	1	0	1	0	0
AUS9	AUS9-Go	1	1	0	1	0	0
AUS9	AUS9-Gf	1	1	0	1	0	0

1: PCR product was obtained. 0: no PCR product was obtained. 1L: large PCR product was obtained. NA: not applicable. *Cam.*: *Campylobacter*. Intestinal isolates are highlighted in blue.

Participant code	Isolate code	*Cam*. spp primers	Pcisus primers	CON1 primers	CON2 primers	VI primers	ZoT primers
AUS9	AUS9-Gm	1	1	0	1	0	0
AUS9	AUS9-Gs	1	1	0	1	0	0
AUS9	AUS9-Gt	1	1	0	1	0	0
AUS9	AUS9-Gx	1	1	0	1	0	0
AUS10	AUS10-Ga	1	1	0	1	1L	0
AUS10	AUS10-Gb	1	1	0	1	0	0
AUS10	AUS10-Gc	1	1	0	1	0	0
AUS10	AUS10-Gd	1	1	0	1	0	0
AUS10	AUS10-Ge	1	1	0	1	0	0
AUS10	AUS10-Gf	1	1	0	1	0	0
AUS10	AUS10-Gm	1	1	0	1	1L	0
AUS10	AUS10-Gn	1	1	0	1	0	0
AUS12	AUS12-Ga	1	1	1	0	NA	0
AUS12	AUS12-Gb	1	1	1	0	NA	0
AUS12	AUS12-Gc	1	1	1	0	NA	0
AUS12	AUS12-Gd	1	1	1	0	NA	0
AUS12	AUS12-Ge	1	1	1	0	NA	0
AUS12	AUS12-Gf	1	1	0	1	0	0
AUS13	AUS13-Ga	1	1	1	0	NA	0
AUS13	AUS13-Gb	1	1	1	0	NA	0
AUS13	AUS13-Gc	1	1	1	0	NA	0
AUS13	AUS13-Gd	1	1	1	0	NA	0
AUS13	AUS13-Gf	1	1	1	0	NA	0
AUS13	AUS13-Gm	1	1	1	0	NA	0
AUS13	AUS13-Gk	1	1	1	0	NA	0
AUS13	AUS13-Gn	1	1	1	0	NA	0
AUS13	AUS13-Ge	1	1	1	0	NA	0
AUS14	AUS14-Ga	1	1	1	0	NA	0
AUS14	AUS14-Gb	1	1	1	0	NA	0
AUS14	AUS14-Gc	1	1	1	0	NA	0
AUS14	AUS14-Gd	1	1	1	0	NA	0
AUS15	AUS15-Ga	1	1	1	0	NA	0
AUS15	AUS15-Ga1	1	1	1	0	NA	0
AUS15	AUS15-Gb	1	1	1	0	NA	0
AUS15	AUS15-Gc	1	1	1	0	NA	0
AUS15	AUS15-Ge	1	1	1	0	NA	0
AUS15	AUS15-Gd	1	1	1	0	NA	0
AUS15	AUS15-Gm	1	1	1	0	NA	0
AUS 15	AUS15-Ga	1	1	1	0	NA	0
AUS15	AUS15-Gn	1	1	1	0	NA	0
AUS15	AUS15-Gf	1	1	1	0	NA	0
AUS15	AUS15-Gk	1	1	1	0	NA	0
AUS15	AUS15-Go	1	1	1	0	NA	0
AUS19	AUS19-GA	1	1	1	0	NA	0
AUS19	AUS19-Gb	1	1	0	1	0	0
AUS19	AUS19-Gc	1	1	0	1	0	0

1: PCR product was obtained. 0: no PCR product was obtained. 1L: large PCR product was obtained. NA: not applicable. *Cam.*: *Campylobacter*. Intestinal isolates are highlighted in blue.

Participant code	Isolate code	*Cam*. spp primers	Pcisus primers	CON1 primers	CON2 primers	VI primers	ZoT primers
AUS19	AUS19-Gd	1	1	0	1	0	**0**
AUS19	AUS19-Ge	1	1	0	1	0	**0**
AUS19	AUS19-Gn	1	1	0	1	0	**0**
AUS19	AUS19-Gm	1	1	0	1	0	**0**
AUS19	AUS19-Gf	1	1	0	1	0	**0**
AUS20	AUS20-Ga	1	1	1	0	NA	**0**
AUS20	AUS20-Gb	1	1	0	1	0	**0**
AUS20	AUS20-Gc	1	1	1	0	NA	**0**
AUS20	AUS20-Gd	1	1	0	1	0	**0**
AUS20	AUS20-Ge	1	1	1	0	NA	**0**
AUS20	AUS20-Gf	1	1	0	1	0	**0**
AUS21	AUS21-Ga	1	1	0	1	0	**0**
AUS22	AUS22-Ga	1	1	0	1	0	**0**
AUS22	AUS22-Gb	1	1	1	0	NA	**0**
AUS22	AUS22-Gc	1	1	0	1	0	**0**
AUS22	AUS22-Gd	1	1	0	1	0	**0**
AUS22	AUS22-Ge	1	1	1	0	NA	**0**
AUS22	AUS22-Gf	1	1	0	1	0	**0**
AUS22	AUS22-Bd2	1	1	1	0	NA	**0**
AUS23	AUS23-Ga	1	1	0	1	0	**0**
AUS23	AUS23-Gb	1	1	0	1	1	**0**
AUS23	AUS23-Gc	1	1	0	1	0	**1**
AUS23	AUS23-Gd	1	1	1	0	NA	**1**
AUS23	AUS23-Ge	1	1	1	0	NA	**0**
AUS25	AUS25-Ga	1	1	0	1	0	**0**
AUS25	AUS25-Gb	1	1	0	1	0	**0**
AUS25	AUS25-Gc	1	1	0	1	0	**0**
AUS25	AUS25-Gd	1	1	0	1	0	**0**
AUS25	AUS25-Ge	1	1	0	1	0	**0**
AUS25	AUS25-Gf	1	1	0	1	0	**0**
AUS26	AUS26-Ga	1	1	0	1	1L	**1**
AUS26	AUS26-Gb	1	1	0	1	1L	**0**
AUS26	AUS26-Gd	1	1	0	1	1L?	**0**
AUS27	AUS27-Ga	1	1	1	0	NA	**0**
AUS27	AUS27-Gb	1	1	0	1	0	**0**
AUS27	AUS27-Gc	1	1	1	0	NA	**0**
AUS27	AUS27-Gd	1	1	0	1	0	**0**
AUS27	AUS27-Ge	1	1	1	0	NA	**0**
AUS28	AUS28-Ga	1	1	0	1	1	**0**
AUS28	AUS28-Gb	1	1	0	1	1	**0**
AUS28	AUS28-Gc	1	1	0	1	1	**0**
AUS28	AUS28-Gd	1	1	0	1	1	**0**
AUS28	AUS28-Ge	1	1	0	1	1	**0**
AUS28	AUS28-Gf	1	1	0	1	1	**0**

1: PCR product was obtained. 0: no PCR product was obtained. 1L: large PCR product was obtained. NA: not applicable. *Cam.*: *Campylobacter*. Intestinal isolates are highlighted in blue.

Participant code	Isolate code	*Cam*. spp primers	Pcisus primers	CON1 primers	CON2 primers	VI primers	ZoT primers
AUS28	AUS28-Gm	1	1	0	1	1	0
AUS28	AUS28-Gn	1	1	0	1	0	0
AUS29	AUS29-Ga	1	1	1	0	NA	0
AUS29	AUS29-Gb	1	1	0	1	0	0
AUS29	AUS29-Gc	1	1	0	1	0	0
AUS29	AUS29-Gd	1	1	0	1	0	0
AUS29	AUS29-Ge	1	1	0	1	0	0
AUS30	AUS30-Gc	1	1	1	0	NA	0
AUS30	AUS30-Gd	1	1	0	1	0	0
AUS30	AUS30-Gf	1	1	0	1	0	0
AUS31	AUS31-GA	1	1	1	0	NA	1
AUS31	AUS31-GB	1	1	1	0	NA	1
AUS32	AUS32-Ga	1	1	0	1	1	0
AUS32	AUS32-Gb	1	1	0	1	1	0
AUS32	AUS32-Gc	1	1	0	1	1	0
AUS32	AUS32-Gd	1	1	0	1	1	0
AUS32	AUS32-Ge	1	1	0	1	1	0
AUS32	AUS32-Gf	1	1	0	1	1	0
AUS33	AUS33-Ga	1	1	0	1	1	0
AUS33	AUS33-Gb	1	1	0	1	1	0
AUS33	AUS33-Gc	1	1	0	1	1	0
AUS33	AUS33-Gd	1	1	0	1	1	0
AUS33	AUS33-Ge	1	1	0	1	1	0
AUS33	AUS33-Gf	1	1	0	1	1	0
AUS33	AUS33-Gm	1	1	0	1	1	0
AUS34	AUS34-Ga	1	1	1	0	NA	0
AUS34	AUS34-Gb	1	1	1	0	NA	0
AUS34	AUS34-Gc	1	1	1	0	NA	0
AUS34	AUS34-Gd	1	1	1	0	NA	0
AUS34	AUS34-Ge	1	1	1	0	NA	0
AUS35	AUS35-Ga	1	1	1	0	NA	0
AUS35	AUS35-Gb	1	1	1	0	NA	0
AUS35	AUS35-Gc	1	1	1	0	NA	0
AUS35	AUS35-Gd	1	1	1	0	NA	0
AUS37	AUS37-Ga	1	1	1	0	NA	0
AUS39	AUS39-Ga	1	1	0	1	0	0
AUS39	AUS39-Gb	1	1	0	1	0	0
AUS39	AUS39-Gc	1	1	0	1	0	0
AUS39	AUS39-Gd	1	1	0	1	0	0
AUS39	AUS39-Ge	1	1	0	1	0	0
AUS39	AUS39-Gm	1	1	0	1	0	0
AUS40	AUS40-Ga	1	1	1	0	NA	0
AUS42	AUS42-Ga	1	1	0	1	0	0
AUS42	AUS42-Gb	1	1	0	1	0	0

1: PCR product was obtained. 0: no PCR product was obtained. 1L: large PCR product was obtained. NA: not applicable. *Cam.*: *Campylobacter*. Intestinal isolates are highlighted in blue.

Recruit code	Isolate code	*Cam*. spp primers	Pcisus primers	CON1 primers	CON2 primers	VI primers	ZoT primers
AUS43	AUS43-Ga	1	1	1	0	NA	0
AUS43	AUS43-Gc	1	1	0	1	0	0
AUS43	AUS43-Ge	1	1	1	0	NA	0
AUS43	AUS43-Gm	1	1	1	0	NA	0
AUS43	AUS43-Gn	1	1	1	0	NA	0
AUS43	AUS43-Go	1	1	1	0	NA	0
AUS43	AUS43-Gk	1	1	1	0	0	0
AUS44	AUS44-Ga	1	1	0	1	1L	0
AUS44	AUS44-Gb	1	1	0	1	1L	0
AUS44	AUS44-Gc	1	1	0	1	1L	0
AUS45	AUS45-Ga	1	1	0	1	1	0
AUS45	AUS45-Gb	1	1	0	1	1	0
AUS45	AUS45-Gc	1	1	0	1	1	0
AUS45	AUS45-Gd	1	1	0	1	1	0
AUS45	AUS45-Ge	1	1	0	1	1	0
AUS45	AUS45-Gf	1	1	0	1	1	0
AUS46	AUS46-Ga	1	1	1	0	NA	0
AUS46	AUS46-Gb	1	1	1	0	NA	0
AUS46	AUS46-Gc	1	1	1	0	NA	0
AUS47	AUS47-Ga	1	1	1	0	NA	1
AUS47	AUS47-Gb	1	1	1	0	NA	1
AUS47	AUS47-Gc	1	1	0	1	1	0
AUS47	AUS47-Sa	1	1	0	1	0	0
AUS48	AUS48-Ga	1	1	0	1	0	0
AUS48	AUS48-Gb	1	1	0	1	0	0
AUS48	AUS48-Gd	1	1	0	1	0	0
AUS48	AUS48-Ge	1	1	0	1	0	0
AUS48	AUS48-Gf	1	1	0	1	0	0
AUS48	AUS48-Ba	1	1	0	1	0	0
AUS49	AUS49-Ga	1	1	0	1	1	0
AUS49	AUS49-Gb	1	1	0	1	0	0
AUS50	AUS50-Gb	1	1	1	0	NA	0
AUS50	AUS50-Gc	1	1	1	0	NA	0
AUS50	AUS50-Gd	1	1	0	1	0	0
AUS50	AUS50-Ge	1	1	1	0	NA	0
AUS50	AUS50-Gf	1	1	1	0	NA	0
AUS51	AUS51-Ga	1	1	0	1	0	0
AUS51	AUS51-Bd	1	1	0	1	1L	0
AUS52	AUS52-Ga	1	1	0	1	0	0
AUS52	AUS52-Gb	1	1	0	1	0	0
AUS52	AUS52-Gc	1	1	0	1	0	0
AUS52	AUS52-Gd	1	1	0	1	0	0
AUS52	AUS52-Ge	1	1	0	1	0	0
AUS52	AUS52-Gf	1	1	0	1	0	0

1: PCR product was obtained. 0: no PCR product was obtained. 1L: large PCR product was obtained. NA: not applicable. *Cam.*: *Campylobacter*. Intestinal isolates are highlighted in blue.

Appendix XXII: Alignments of the *zot* gene sequences for *C. concisus* obtained from clinical isolates.

```
Alignment:    Global DNA alignment against reference molecule
Parameters:   Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)

   Reference molecule:  13826, Region 1 to 966
   Number of sequences to align:  10
   Total length of aligned sequences with gaps:  966 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
   Ref:  13826              1 to     966    (   966 bps)      --
     2:  UNSW3              1 to     966    (   966 bps)      89%
     3:  UNSWCS             1 to     966    (   966 bps)      88%
     4:  RCH6               1 to     966    (   966 bps)     100%
     5:  RCH11              1 to     966    (   966 bps)      89%
     6:  RCH26              1 to     966    (   966 bps)      87%
     7:  AUS7-Ga            1 to     966    (   966 bps)      88%
     8:  AUS23-Gc           1 to     966    (   966 bps)      97%
     9:  AUS23-Gd           1 to     966    (   966 bps)      87%
    10:  AUS26-Ga           1 to     966    (   966 bps)      94%


13826             1 aactctttcaaattctgaattacaagaaacaaagtaatcaatgtagttgcctgacttctt
UNSW3             1 aacccttttcaaaatctgcatggcaagaaacaaagtaatcaatgtagttgcctgacttctt
UNSWCS            1 aacccttttcaaaatctgcatggcaagaaacaaagtaatcaatgtagttgcctgacttctt
RCH6              1 aactctttcaaattctgaattacaagaaacaaagtaatcaatgtagttgcctgacttctt
RCH11             1 aacccttttcaaaatctgcatggcaagaaacaaagtaatcaatgtagttgcctgacttctt
RCH26             1 aactctttcaaaatctgcatggcaagaaacaaaataatcaatgtaattgcctgacttctt
AUS7-Ga           1 aactctttcaaaatctgcatggcaagaaacaaaataatcaatgtagttgcctgacttctt
AUS23-Gc          1 aactctttcaaattctgcgttacaagaaacaaagtaatcaatgtagttgcctgacttctt
AUS23-Gd          1 aactctttcaaaatctgcatggcaagaaacaaaataatcaatgtagttgcctgacttctt
AUS26-Ga          1 aacccttttcaaaatctacatggcaagaaacaaagtaatcaatgtagttgcctgacttctt

13826            61 atcatgtaagaatatatggcagttagaagaagaaagaagctccaaaaagctatctaaaga
UNSW3            61 atcttgtaagaatatatggcagtttgagaaagaaaggagttctaagaagctatctaaaga
UNSWCS           61 atcttgtaagaatatatggcagtttgagaaagaaaggagttctaagaagctatctaaaga
RCH6             61 atcatgtaagaatatatggcagttagaagaagaaagaagctccaaaaagctatctaaaga
RCH11            61 atcttgtaagaatatatggcagtttgagaaagaaaggagttctaagaagctatctaaaga
RCH26            61 atcttgtaagaatatatggcagtttgaaaaagaaagaagttctaagaagctatctaaaga
AUS7-Ga          61 atcttgtaagaatatatggcagtttgagaaagaaagaagttctaagaagctatctaaaga
AUS23-Gc         61 atcatgtaagaatatatggcagttagaagaagaaagaagctccaaaaagctatctaaaga
AUS23-Gd         61 atcttgtaagaatatatggcagtttgagaaagaaagaagttctaagaagctatctaaaga
AUS26-Ga         61 atcttgtaagaatatatggcagtttgagaaagaaagaagttctaagaagctatctaaaga

13826           121 taaatcaatagcgtaatttctaaatttacaaccgcttggaaaacaagttatccttagata
UNSW3           121 taaatcaatggcgtaatttctaaatttacaaccgcttggaaagcaagttacccttagata
UNSWCS          121 taaatcaatggcgtaatttctaaatttacaaccgcttggaaagcaagttacccttagata
RCH6            121 taaatcaatagcgtaatttctaaatttacaaccgcttggaaaacaagttatccttagata
RCH11           121 taaatcaatggcgtaatttctaaatttacaaccgcttggaaagcaagttacccttagata
RCH26           121 taaatcaatggcataatttctaaatttacaaccatttggaaagcaagttatctttagata
AUS7-Ga         121 taaatcaatggcgtaatttctaaatttacaaccatttggaaagcaagttatctttagata
AUS23-Gc        121 taaatcaatggcgtaatttctaaatttacaaccgcttggaaaacaagttatccttagata
AUS23-Gd        121 taaatcaatggcgtaatttctaaatttacaaccatttggaaagcaagttatctttagata
AUS26-Ga        121 taaatcaatggcgtaatttctaaatttacaaccgcttggaaagcaagttatccttagata

13826           181 gattttattatcgttaaaaatagtggtattaatatctgatttgtctgcatcgtttgaaat
UNSW3           181 gattctattattattaaaaatagtggtgtttatatctgaattatctacactatttgaaat
UNSWCS          181 gattctattattattaaaaatagtggtgtttatatctgaattatctacactatttgaaat
RCH6            181 gattttattatcgttaaaaatagtggtattaatatctgatttgtctgcatcgtttgaaat
RCH11           181 gattctattattattaaaaatagtggtgtttatatctgaattatctacactatttgaaat
RCH26           181 gattctattgttattaaaaatagtggtgtttatatctgaattatctacgctatttgaaat
AUS7-Ga         181 gattctattactgttaaaaatagtggtgtttatatctgaattatccacgctatttgaaat
AUS23-Gc        181 gattttattatcgttaaaaatagtggtgttaatatctgatttgtctgcatcgtttgaaat
AUS23-Gd        181 gattctattgttattaaaaatagtggtgtttatgtctgaattatctacgctatttgaaat
AUS26-Ga        181 gattctattgttattaaaaatagtggtgtttatatctgaattatctacactatttgaaat
```

```
13826          241 agcttttatattcttagaatctgaagcatttaaatcaacaaatctagtttcttgtttagt
UNSW3          241 tgttttaggctctttagaatcagaagtatttaaatcaacaaacctagcttcttgtttaat
UNSWCS         241 tgttttaggctctttagaatcagaagtatttaaatcaacaaatctagtatcttgaatagt
RCH6           241 agcttttatattcttagaatctgaagcatttaaatcaacaaatctagtttcttgtttagt
RCH11          241 tgttttaggctctttagaatcagaagtatttaaatcaacaaatctagtatcttgaatagt
RCH26          241 tgttttaggcttttagaatcagaagtatttaaatcaacaaacctagtatcttgactagt
AUS7-Ga        241 tgttttaggtgttttagaatcagaagtatttaaatcaacaaacctagtatcttgactaga
AUS23-Gc       241 agcttttatattcttagaatctgaagcatttaaatcaacaaacctagtttcttgtttagt
AUS23-Gd       241 tgttttaggcttttagaatcagaagtatttaaatcaacaaacctggtatcttgaatagt
AUS26-Ga       241 tgttttaggctctttagaatcagaagtatttaaatcaacaaacctagtatcttgtttagt

13826          301 tgattgagctggctcatgcttaggctctaaaaatttataaaccacataagaaaaaactat
UNSW3          301 tgattgagcttgatcatgtttaggctctaagaatttatatatagcgtaagaagctacaat
UNSWCS         301 tgattgagctggctcatgtttaggctctaagaatttatatatagcgtaagaagctacaat
RCH6           301 tgattgagctggctcatgcttaggctctaaaaatttataaaccacataagaaaaaactat
RCH11          301 tgattgagctggctcatgtttaggctctaagaatttatatatagcgtaagaagctacaat
RCH26          301 tgattgagctggctcatgcttaggctctagaaatttatatatagcgtaagaagctataat
AUS7-Ga        301 tgactgatctggctcatgcttaggctctaaaaatttataaagcaaataagaaaaaacaat
AUS23-Gc       301 tgattgagctggctcatgcttaggctctaaaaatttataaaccacataagaaaaaaccat
AUS23-Gd       301 tgattgagctggctcatgcttaggctctagaaatttatatatagcgtaagaagctacaat
AUS26-Ga       301 tgattgagctggctcatgtttaggctctaagaatttataaaccaaataagaaaaaactat

13826          361 gaaagctaataaaaataaaatctttttagttgcatagcttttataaatttcattagaacc
UNSW3          361 aaaaattattaaaaataaaatctttttagttgcatagcttttataaatttcttttgaacc
UNSWCS         361 aaaaattattaaaaataaaatctttttagttgcatagcttttataaatttcttttgaacc
RCH6           361 gaaagctaataaaaataaaatctttttagttgcatagcttttataaatttcattagaacc
RCH11          361 aaaaattattaaaaataaaatctttttagttgcatagcttttataaatttcttttgaacc
RCH26          361 aaaaattattaaaaataagatctttttagttgcatagcttttataaatttcttttgagcc
AUS7-Ga        361 aaaagccaataaaaataagatttttttagtcgcatagcttttataaatttcttttgagcc
AUS23-Gc       361 gaaagctaataaaaataaaatctttttagttgcatagcttttataaatttcattagatcc
AUS23-Gd       361 aaaaattaataaaaataagatctttttagttgcatagcttttataaatttcttttgagcc
AUS26-Ga       361 gaaagccaataaaaataaaatctttttagttgcatagcttttataaatttcattagatcc

13826          421 actactatataaatttgatatttttgattaaatttcaaattctcagaattaataaggtt
UNSW3          421 actactatataaatctgagacttttgattaaattttagattttcagaacgaataagatt
UNSWCS         421 actactatataaatctgaaacttttgattaaattttagattttcagaacgaataagatt
RCH6           421 actactatataaatttgatatttttgattaaatttcaaattctcagaattaataaggtt
RCH11          421 actactatataaatctgaaacttttgattaaattttagattttcagaacgaataagatt
RCH26          421 actactatataaatctgatacttttgattaaattttagattttcagagcgaataagatt
AUS7-Ga        421 actactatataaatctgatacttttgattaaattttagattttcagagcgaataagatt
AUS23-Gc       421 actactatataaatttgatatttttgattaaatttcaaattctcagaattaataaggtt
AUS23-Gd       421 actactatataaatctgatacttttgattaaattttagattttcagagcgaataagatt
AUS26-Ga       421 actactatataaatttgatatttttgattaaatttcaaattctcagaattaataaggtt

13826          481 atcatttacctgagatgaagtgctataaactttatacttaaaaagcttgctaaaaagcct
UNSW3          481 gtaattaaaattagatgaagtgctatagactttatatttaaaaagactactaaaaagcct
UNSWCS         481 gtaattaaaattagatgaagtgctatagactttatatttaaaaagactactaaagagcct
RCH6           481 atcatttacctgagatgaagtgctataaactttatacttaaaaagcttgctaaaaagcct
RCH11          481 gtaattaaaattagatgaagtgctatagactttatatttaaaaagactactaaaaagcct
RCH26          481 ataattaaaattagatgaagtgctataaactttatatttaaaaagactactaaagagcct
AUS7-Ga        481 ataattaaaattagatgaagtgctataaactttatatttaaaaagactactaaaaagcct
AUS23-Gc       481 atcatttacctgcgatgaagtgctataaactttatacttaaaaagcttgctaaaaagcct
AUS23-Gd       481 ataattaaaattagatgaagtgctataaactttatatttaaaaagactactaaagagcct
AUS26-Ga       481 atcatttacctgcgatgaagtgctataaactttatacttaaaaagcttgctaaaaagcct

13826          541 tttaccgctaggctgagccatatacattaattcagtatgaactaaatactctctatttgt
UNSW3          541 tttaccactaggctgagccatatacattaattcagtatgaactaaatattctctatttgt
UNSWCS         541 tttgccactaggctgagccatatacattaattcagtatgaactaaatactctctatttgt
RCH6           541 tttaccgctaggctgagccatatacattaattcagtatgaactaaatactctctatttgt
RCH11          541 tttgccgctaggctgagccatatacattaactcggtatgaacgagatactctctatttgt
RCH26          541 tttgccactaggctgggccatatacattaattcagtatgaactaaatattctctatttgt
AUS7-Ga        541 tttaccactaggctgggccatatacattaattcagtatgaactaaatattctctatttgt
AUS23-Gc       541 tttaccgctaggctgagccatatacattaattcagtatgaacgagatattctctatttgt
AUS23-Gd       541 tttgccactaggctgggccatatacattaattcagtatgaactaaatattctctatttgt
AUS26-Ga       541 tttaccgctaggctgagccatatacattaattcagtatgaactaaatactctctatttgt
```

```
13826      601 ctgacgcttagactgaaaaagaaagataatatcaatcccaaaatgtccgtgatagcttaa
UNSW3      601 ctgacgtttcgactggaaaagaaagataatatcaatcccaaaatgtccgtgatagcttag
UNSWCS     601 ttgacgtttagactgaaataaaaagataatatcaatcccaaaatgtccgtgatagcttaa
RCH6       601 ctgacgcttagactgaaaaagaaagataatatcaatcccaaaatgtccgtgatagcttaa
RCH11      601 ttgacgtttagactgaaaaagaaagataatatcaatcccaaaatgtccgtgatagcttaa
RCH26      601 ctgacgtttagactgaaataaaaaaataatatcaatgccaaaatgtccgtgataacttaa
AUS7-Ga    601 ctgacgtttagactgaaataaaaaaataatatcaatgccaaagtgtccgtgataacttaa
AUS23-Gc   601 ctgacgtttagactggaaaagaaagataatatcaatcccaaaatgtccgtgatagcttaa
AUS23-Gd   601 ctggcgtttagactgaaataaaaaaataatatcaatgccaaaatgtccgtgataacttaa
AUS26-Ga   601 ctgacgtttcgactggaaaagaaagataatatcaatcccaaaatgtccgtgatagcttaa

13826      661 aaacctacccaagctatcattaaacgttttagtaaaggtgttataggcttcatcgagaac
UNSW3      661 aaacctacccaagctttcattaaaggttttagtaaaggtgttgtaagcttcatcaagaac
UNSWCS     661 aaatctacccaagctatcattaaagttttcgtaaaggtgttgtaagcttcatcaagaac
RCH6       661 aaacctacccaagctatcattaaacgttttagtaaaggtgttataggcttcatcgagaac
RCH11      661 aaacctacccaagctctcattaaacgttttcgtgaaggtgttgtaagcttcatcaagaac
RCH26      661 aaacctaccaagactatcattaaatgtcttagtaaaggtgttgtaagcttcatcaagaac
AUS7-Ga    661 aaatctacccaagctatcgttaaatgtttttgtaaaggtgttgtaagcttcatcaagaac
AUS23-Gc   661 aaacctacccaagctatcattaaacgttttagtaaaggtgttataagcttcatcaaggac
AUS23-Gd   661 aaacctacccagactatcattaaatgttttagtaaaggtgttgtaagcttcatcaagaac
AUS26-Ga   661 aaacctacccaagctatcattaaacgttttagtaaaggtgttataagcttcatcgagaac

13826      721 tattaaacaatgatgataattttcatatatgccacttttaaggcatattcatcataatt
UNSW3      721 tatcaagcaatgatgataattttcgtaaattccatttttcaaggcatattcatcataatt
UNSWCS     721 tattaaacaatgatgataattttcgtaaattccatttttcaaggcatattcatcataatt
RCH6       721 tattaaacaatgatgataattttcatatatgccacttttaaggcatattcatcataatt
RCH11      721 tattaagcaatgatgataattttcatatatgccacttttaaggcatattcatcataatt
RCH26      721 tatcaagcaatgatgataattttcatagatgccatttttaaagcatattcatcataatt
AUS7-Ga    721 tatcaagcaatgatgataattttcgtatatgccatttttaaagcatattcatcataatt
AUS23-Gc   721 tattaaacaatgatgataattttcatatatgccacttttaaggcatattcatcataatt
AUS23-Gd   721 tattaagcaatgatgataattttcatagatgccatttttaaagcatattcatcataatt
AUS26-Ga   721 tattaaacaatgatgataattttcatatatgccacttttaaggcatattcatcatagtt

13826      781 atctacattgtctaaaaagccattttcgtattgagaactaagcgtatattcttgactaac
UNSW3      781 atctacattctctaaaaagccattttcatattgagaactaagggtatattcttgactaac
UNSWCS     781 atctacattctctaaaaagccattttcgtattgagaactaagcgtatattcttgactaac
RCH6       781 atctacattgtctaaaaagccattttcgtattgagaactaagcgtatattcttgactaac
RCH11      781 atctacattatctaaaaagccattttcgtattgagaactaagcgtatattcttgactaac
RCH26      781 atctacattatctaaaaagccattttcatattgagaactaagcgtatattcttgactaac
AUS7-Ga    781 atctacattatctaaaaagccattttcatattgagagttaagcgaatattcttgactaac
AUS23-Gc   781 atctacattgtctaaaaaaccattttcgtattgagaactaagcgtatattcttggctaac
AUS23-Gd   781 gtctatattatctaaaaagccattttcatattgagaactaagggtgtattcttgactaac
AUS26-Ga   781 atctacattgtctaaaaagccattttcgtattgagaactaagcgtatattcttgactaac

13826      841 agcagtaagaaaatcatttttatcatattgttttacaaagccatcaaaatgatcaaattt
UNSW3      841 cgcagtaagaaagtcatttttatcatattgttttacaaagccgtcaaaatgatcaaattt
UNSWCS     841 cgcagtaagaaaatcatttttatcatattgttttacaaagccgttaaaatgatcaaattt
RCH6       841 agcagtaagaaaatcatttttatcatattgttttacaaagccatcaaaatgatcaaattt
RCH11      841 cgcagtaagaaaatcatttttatcatattgctttacaaagccgttaaaatgatcaaattt
RCH26      841 agcggtaagaaaatcatttttatcatattgctttacaaagccatcaaaatgatcaaattt
AUS7-Ga    841 cgcagtaagaaaatcattcttatcatattgctttacaaagccatcaaaatgatcaaattt
AUS23-Gc   841 cgcagtaagaaaatcatttttatcatattgctttacaaagccattaaagagatcaaattt
AUS23-Gd   841 cgcagtaagaaaatcattcttatcatattgctttacaaagccgttaaaatgatcaaattt
AUS26-Ga   841 cgcagtaagaaaatcatttttatcatattgttttacaaagccgtcaaaataatcaaattt

13826      901 taaaccattgatattagtataaataaatctatactttgattcgccttttaaatgcaattc
UNSW3      901 taaaccattgatattagtgtaaataaatctatactttgattcgccttttaagtgcaattc
UNSWCS     901 caagccattaatattagtatagataaatctatactttgattcgccttttaagtgtaattc
RCH6       901 taaaccattgatattagtataaataaatctatactttgattcgccttttaaatgcaattc
RCH11      901 taaaccattgatattagtataaataaatctatactttgattcgccttttaagtgcaattc
RCH26      901 taagccattaatattagtataaataaatctatactttgattcatttttaaatgtagctc
AUS7-Ga    901 taagccgttaatattagtataaataaatctatactttgattcatctttaaatgtaattc
AUS23-Gc   901 taaaccattgatattagtatagataaacctatactttgattcgccttttaagtgcaattc
AUS23-Gd   901 taagccattgatattagtataaataaatctatactttgattcatttttaaatgtagctc
AUS26-Ga   901 taaaccattgatattagtgtaaataaatctatactttgattcgccttttaaatgcaattc
```

```
13826           961 atattc
UNSW3           961 gtattc
UNSWCS          961 gtattc
RCH6            961 atattc
RCH11           961 atatca
RCH26           961 atactc
AUS7-Ga         961 atattc
AUS23-Gc        961 gtattc
AUS23-Gd        961 atactc
AUS26-Ga        961 atattc
```